AN INVESTIGATION OF THE SPECIFICITY AND DIFFERENTIAL ASSAY OF HUMAN PEPSINS

by

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This thesis has been composed by myself and the work presented is my own unless otherwise acknowledged.

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CONTENTS

ABBREVIATIONS

The abbreviations commonly used in this thesis are as follows:

A.P.D.	Acetyl-L-Phenylalanyl-L-diiodotyrosine
Ac.Y.L.V.H.NH_2	Acetyl-L-Tyrosyl-L-Leucyl-L-Valyl-L-Histidyl amide
BIS	N,N′-methylene bisacrylamide
DEAE	diethyl aminoethyl
EDTA	ethylene diamine tetraacetic acid
Hb	haemoglobin
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TEMED	N,N,N′,N′ tetramethylethylenediamine
SMP	slow moving protease
Pg	Pepsinogen

ABSTRACT

Human gastric juice has been resolved into seven distinct protein fractions by chromatography on DEAE-Sephadex A50. Six of these had proteolytic activity against haemoglobin. One of these peaks, peak I, had a relatively high specific activity against Ac.Y.L.V.H.NH_2 and a low specific activity against A.P.D. and was thus identified as gastricsin. Of the other five active peaks, at least three showed high activity against A.P.D. and low activity against Ac.Y.L.V.H.NH_2, while the activity of the remaining two peaks, which were present at extremely low concentrations, was difficult to determine. At least one of the six active peaks has apparently not been isolated previously. Each of the active peaks was electrophoretically homogeneous with respect to activity, so it appears that the peptidase activities are genuine properties of each peak. The seventh peak, which appears not to have been isolated before, was inactive against all substrates used. Experiments indicated that the existence of none of the peaks could be explained by autolysis of other peaks, under the conditions used. Amino acid composition and differences in specific activities indicate that the seven peaks represent distinct proteins.

Studies of the pH optima of hydrolysis of A.P.D. and Ac.Y.L.V.H.NH_2 by two of the pepsins indicate the enzymes have optimal activities at very similar pH values. It may be that a differential assay could be constructed that would determine "total gastricsin-like activity" and "total pepsin-like activity". However, this would not be useful for determining levels of individual pepsins in gastric juice.

It is concluded that, if individual pepsins are to be assayed in unfractionated gastric juice, substrates are needed which are specifically hydrolysed by the pepsins of interest.

Chapter 1 INTRODUCTION

1.1 General Information

Pepsins belong to the Enzyme Commission category E.C. 3.4.23. They are generally described as acid proteinases, because they have maximal activity at acid pH, or as aspartyl or carboxyl proteinases, because of the involvement of such groups in their catalytic mechanism (Tang 1979).

Proteinases of this class have been found in all mammals, fish and birds so far examined, as well as in many micro-organisms (see reviews by Hofmann, 1974 and Morihara, 1974). In the metazoans the enzymes are produced as zymogens which are converted to active enzymes in acid conditions with release of peptides ('activation peptides') at least one of which acts as a pepsin inhibitor (Herriott, 1939). No such zymogens have been found in micro-organisms. As a general rule, the molecular weight of pepsinogens is 40-45,000 and that of pepsins is 30-35,000.

In their primary structures, many areas of homology are found between pepsins from different species (see, e.g. Huang & Tang 1970; Tang et al 1973; Harboe et al 1974; Sepulveda et al 1975). The amino acid sequences around the active site residues Asp-32 and Asp-215 are conserved in each of seven pepsins examined (Hsu et al 1977).

High-resolution X-ray structures have been reported for three aspartyl proteinases. All have similar tertiary structures (Subramanian et al 1977; Hsu et al 1977).

All pepsins are inhibited in stoichiometric ratios by diazoacetyl-DL-norleucine methyl ester (DAN), first used by Rajagopalan et al (1966), and by 1, 2-epoxy-3-nitrophenoxy propane (EPNP), introduced by Tang (1971). Pepsins are also inhibited by pepstatin (Morishima et al 1970), which appears to be a transition state analogue (Marciniszyn et al 1976 a). Pepstatin is a much more potent inhibitor of pepsin-like enzymes than of gastricsin-like enzymes (see below for nomenclature, Sect. 1.2).

Most of the higher animals secrete more than one proteinase of the pepsin-type. Some aspects of this heterogeneity are considered in the next section.

1.2 Heterogeneity of Pepsins and Pepsinogens.

Most early studies were largely based on the determination of pH maxima of activity of pepsin, using various substrates, e.g., Dyckerhoff and Tewes (1933), Geilenkirchen and Elbers (1950), Christensen (1955).

A series of studies by Northrop (1919, 1922) indicated that there are definite pH optima for the combination of casein and egg albumin with swine pepsin. These optima coincide with the pH optima for digestion of the proteins. It thus appeared that different forms of protein substrates have different probabilities of digestion by pepsin. Northrop suggested that pepsin reacts with the ionized form of the substrates.

Fruton and Bergmann (1939) investigated the action of pig pepsin on a variety of synthetic substrates, mostly of the type Z-L-Glu-X, where Z=benzyloxycarbonyl. The compound in which X=L-tyrosine was most readily attacked, with optimum activity at pH4. If either/...

If either carboxyl group in Z-Glu-Tyr was in its amide form pepsin activity was greatly reduced. It was suggested that the ease of hydrolysis of proteins such as casein and edestin could be explained by the presence of many aminodicarboxylic acids and aromatic residues.

Baker (1951) used a new series of substrates with L-Tyr linked to L-Phe. Bonds linking L-amino acids containing benzyl, hydroxybenzyl or diiodohydroxybenzyl side chains were hydrolysed optimally at pH 1·8-2, with Acetyl-L-phenylalanyl-L-diidotyrosine (A.P.D.) being most readily hydrolysed. Compounds in which D-enanticmers were used, in which the ionization of the phenolic group of tyrosine was prevented by acetylation or in which the α-carboxyl group was in its amide form were not hydrolysed.

Taylor (1959 a) found that, with most protein substrates tested, human gastric juice showed maxima of activity at pH 1·6-2 and pH 3·3-4. With Z-Glu-Tyr only one maximum, at pH 4, was found. Extracts of human gastric mucosa behaved in a similar fashion. In these experiments the extent of hydrolysis was measured by direct formol titration, involving reaction of free amino groups with formaldehyde and subsequent titration of carboxyl groups with NaOH; hydrolysed peptide bonds were thus estimated quantitatively (Taylor 1957).

Using purified pig pepsin Taylor (1959 b) studied interactions between enzyme and substrate. The pepsin was homogeneous by paper electrophoresis, and it digested several proteins with two pH maxima around pH 1·8-2 and pH 4. Using salt fractionation and/...

and bulk adsorption onto ion-exchange resins Taylor could not separate the activities causing these maxima, and he concluded that a single enzyme has two types of active site which attack protein substrates with different pH maxima. However, in the light of subsequent work which has revealed the multiplicity of pepsins it would appear that these separation methods were too crude to be effective.

It is notoriously difficult to evaluate data for pH dependence of proteinase action on protein substrates because of the range of influencing factors, e.g., state of the substrate (native or denatured), number and variety of scissile bonds etc. It is clear from the work of Fruton and Bergmann (1939) and Baker (1951), mentioned earlier, that pig pepsin has different pH optima depending on the nature of the substrate. Thus, the existence of pH maxima for the digestion of protein substrates may be explained on the basis of hydrolysis of different types of peptide bonds. It may be noted that in the experiments in which Taylor used a synthetic substrate containing only one scissile bond only one pH maximum was observed (Taylor 1959 a).

The introduction of ion-exchange chromatography made the separation of enzymatic fractions much easier. Minor porcine pepsins, parapepsins I and II, (later re-named pepsins B and C respectively) were isolated by chromatography on DEAE-cellulose along with the major pepsin, pepsin A (Ryle and Porter 1959). Pepsins B and C were thus named because of their order of elution. Pepsin A has been characterised as the most abundant pepsin/....

pepsin in the pig stomach. It has relatively high activity against several synthetic substrates, e.g. A.P.D., and against protein substrates, and is able to clot milk (Ryle and Porter 1959; Ryle 1970). It is typical of these pepsins that the ratio of glutamate: aspartate (each including the amide) is below 1·0, and that of leucine: isoleucine is below 1·5. Pig pepsin A contains one residue of phosphate per molecule, bound at serine - 68 (Tang & Hartley 1970). Neumann et al (1969), using the phenol-sulphuric acid test, detected about three molar equivalents of neutral sugar per mole of pig pepsinogen, using four different batches of zymogen. No sugar was found in pepsin formed from these pepsinogen samples. It was shown that acid treatment liberated this covalently-bound sugar. Further, it was suggested that release of carbohydrate precedes release of activation peptides from pepsinogen. However, Stepanov et al (1971) using the same phenol-sulphuric acid test as well as gas-liquid chromatography of trimethylsilyl derivatives found no significant levels of sugar in pig pepsinogen. The absence of covalently-bound sugar in pig pepsinogen has been confirmed by Kay and Dykes (1977).

Porcine pepsin B is distinguished by its ability to hydrolyse small peptides and by its low milk-clotting activity and its apparent inability to hydrolyse haemoglobin (Ryle 1960: Ryle 1965). No enzyme of this type has been identified in humans.

Pepsin C has been characterised by its relatively low activity against A.P.D. (Ryle & Porter 1959); it can hydrolyse protein substrates. A new synthetic substrate, Acetyl-L-tyrosyl-L-leucyl-L-valyl-L-histidyl amide (Ac.Y.L.V.H.NH_2), has been produced/.....

produced (Auffret & Ryle 1979); it is selectively hydrolysed at the Tyr-Leu bond by pepsin C, not by pepsin A, under the conditions used (Ryle & Auffret 1979). Porcine pepsin C has high ratios of glutamate: aspartate (including amides) and leucine: isoleucine compared to those of pepsin A.

A further porcine enzyme, pepsin D, was isolated, as were all the zymogens (Ryle 1960, 1965; Lee & Ryle 1963). It has been shown that pepsin D is identical to dephosphorylated pepsin A (Lee & Ryle 1967), their electrophoretic mobilities being the same. Amino acid compositions and specific activities of pepsins A and D are virtually the same.

from human gastric juice

Two proteolytic components were separated on Amberlite IRC-50, a cationic ion-exchanger (Richmond et al 1958). They were named pepsin and gastricsin. The human pepsin can be correlated with pig pepsin A by virtue of its activity against haemoglobin and A.P.D. It is the most abundant proteinase in the human stomach. Gastricsin has very low, if any, activity against A.P.D. (Chiang et al 1966) but high activity against haemoglobin; it is therefore considered analagous to pig pepsin C.

Three peaks obtained from human stomachs by chromatography on DEAE-cellulose at pH 5·3 were studied by Seijffers et al (1963 a). Extracts of fundic mucosa yielded all three peaks, while pyloric and duodenal mucosae yielded only one, this being Pg I, the first to be eluted from the column and thus presumably the least negatively charged. The authors assumed that the peaks represented zymogens although at pH 5·3 some activation to pepsins might/.....

might have occurred. In a further paper, Seijffers et al (1963 b) followed the activation of zymogens. Pepsinogen II produced pepsins IIA and IIB. However, pepsin IIB was chromatographically indistinguishable from pepsin III, so clearly the Pg II may have contained some Pg III.

The homogeneity of these pepsinogens was questioned by Turner et al (1970). Seven fractions were obtained by agar gel electrophoresis, one of which was not inactivated by acidification and neutralization. Acidification of pepsinogen causes its activation to pepsin by limited proteolysis, with concomitant release of a peptide which inhibits pepsin (Herriott 1939). Subsequent neutralization of such an activated sample will destroy peptic activity (Seijffers et al 1964). Inactivation by this treatment is considered a definitive test for pepsinogen. The fraction not destroyed this way was therefore not regarded as a pepsinogen. However, chicken pepsins are considerably more alkali-stable, retaining activity at pH 8 under certain conditions (Bohak 1969).

Samloff (1969) subjected extracts of human gastric mucosa to agar gel electrophoresis and located eight zones of potential activity. One of these was not inactivated by acidification and subsequent neutralization and was named 'slow-moving protease' (SMP). This appears to be the equivalent of activated P IV detected by Kushner et al (1964).

Etherington and Taylor (1969) obtained eight proteolytic zones from human gastric juice, using agar gel electrophoresis. One zone occurred only in achlorhydric samples; this zone, zone 6, was eliminated/.....

eliminated by preliminary incubation at pH 2 and was probably a pepsinogen. Zone 4 disappeared after incubation at pH 3·5, the pH of optimum digestion of pig pepsin inhibitor (Seijffers et al 1964); it was thought zone 4 represented a pepsin-inhibitor complex. Only zones 3 and 5 occurred in all 50 samples. No significant difference was found in secretion between healthy subjects and those with gastric disease symptoms. These authors also fractionated human gastric juice on Amberlite IRC-50 and on DEAE-cellulose. Two peaks were obtained on Amberlite, similar to those described by Richmond et al (1958); one peak ('pepsin') contained mainly electrophoresis zone 3 and the other ('gastricsin') contained mainly zone 5. Chromatography on DEAE-cellulose gave five peaks of activity. Thus it is clear that chromatography on Amberlite IRC-50 does not reveal the full complexity of the mixture.

Electrophoresis of extracts of human gastric mucosa produced several zones (Etherington & Taylor 1970). One of these, zone 7, corresponded to P IV of Kushner et al (1964). Activated extracts of pyloric mucosa showed most of their activity at zone 5, the least electronegative zone. Also, chromatography of such extracts on Amberlite IRC-50 yielded only one peak, that of gastricsin.

The SMP was resolved electrophoretically into two components (Etherington & Taylor 1972). Roberts and Taylor (1978 b) isolated the major enzyme and called it proteinase 2.

Whitecross et al (1973) located six major and four minor bands of potential proteinase activity in gastric mucosa, using agar/.....

agar gel electrophoresis. All of the six major bands were present in extracts of fundic mucosa, and only one was present in duodenal mucosa, this band being electrophoretically equivalent to the least electronegative of the fundic bands. The authors also showed that extra bands could be identified if a high voltage (40 v/cm) was used rather than 10 v/cm.

Roberts and Taylor (1978 a) have reported the purification in relatively large amounts of four of their pepsin fractions by repeated chromatography on DEAE-cellulose. These pepsins were shown to be homogeneous on agar gel and SDS-polyacrylamide gel electrophoresis. Although this purification procedure is rather complicated, it represents the most successful scheme yet used for large-scale preparation of the minor human pepsins.

As has been shown, improvement in type and quality of ion-exchange resins has led to improved separation of pepsins, as may be seen by comparison of the results obtained by Richmond et al (1958) and Roberts and Taylor (1978 a). Similarly, many different electrophoretic systems have been used and different results have been obtained. As Whitecross et al (1973) showed, a simple change in voltage can produce considerable differences in resolution.

Thus, because of these factors, nomenclature of pepsins and pepsinogens has become confused as different authors have devised their own systems. Table 1 shows a correlation, as far as possible, using the pig pepsin nomenclature, suggested by Ryle and accepted by the International Union of Biochemistry, as the basis. The correlations shown can be justified for the major pepsins/.....

pepsins on the basis of activity against various substrates. It should be appreciated that it is difficult to place some of the minor enzymes in the scheme shown. Some of the nomenclatures rely on order of elution from ion-exchange columns, others rely on electrophoretic mobility. Further evidence for the sub-division of pepsins, based on cellular distribution and immuno-chemistry, is discussed in the following sections.

Ryle (1965) (pig)	Richmond et al (1958)	Seijffers et al (1963 a)	Kushner et al (1964)	Samloff (1969)	Etherington & Taylor (1969, 1970)
Pepsin (pepsinogen) A E.C.3.4.23.1	Pepsin	pepsinogens Pg II,III	pepsinogens P I, II	Group I pepsinogens (Pg 1-5)	pepsin 3 (also probably 1,2,3a)
Pepsin (pepsinogen) C E.C.3.4.23.3	gastricsin	Pg I	P III	group II pepsinogens (Pg 6,7)	pepsin 5
Pepsin (pepsinogen) B E.C. 3.4.23.2					
Pepsin (pepsinogen) D					
			P IV	SMP	zone 7 (Proteinase 2, Roberts & Taylor 1978 b)

1.3 Secretion of Pepsinogens

1.3.1 Physiology of the Stomach

The mammalian stomach is divided in three areas:-

(a) Cardiac Area

This occurs directly after the junction of the oesophagus and the stomach. It contains mainly mucus-secreting cells.

(b) Fundus and Body

This is the major part of the human stomach. The glands are termed 'oxyntic glands', which consist of three types of cells:-

(i) neck, or mucous neck, cells which produce mainly glycoproteins plus some pepsinogen.

(ii) chief cells, which produce pepsinogen.

(iii) parietal cells, which secrete acid.

(c) Pylorus (Antrum)

The pylorus is the part of the stomach adjoining the duodenum, via the pyloric sphincter. The pylorus has a different shape and composition of glands from those of the fundus. Brünners' glands also are found in the pylorus as well as in the duodenum. It should be noted that there are no parietal cells in the pylorus or duodenum.

1.3.2 Sites of Secretion of Pepsinogens.

The work of Seijffers et al (1963 a), Etherington and Taylor (1970)/....

(1970) and Whitecross et al (1973) has already been mentioned (Section 1.2).

Samloff (1969) examined extracts of mucosa from different parts of the human stomach and duodenum. Samples were obtained at surgery. Eight electrophoretic zones, proteolytically-active after acidification, were located. Seven were pepsinogens (Pg 1-7 in order of decreasing electronegativity) and one was SMP. Pepsinogens 1-5 were found in mucosa from the fundus and body of the stomach only, while Pg 6 and 7 were found in mucosa throughout the stomach and proximal duodenum, as was SMP. Pepsinogens were also found in low concentrations in serum, urine and semen. Those found in serum could presumably arise from resorption of cells of gastric and duodenal mucosa; generally serum contains all seven pepsinogens (Pg 1-7). The zymogens in human semen were studied by Lundquist and Seedorff (1952); preliminary experiments indicated that pepsinogen was formed in the seminal vesicular glands. More recently Chiang et al (1981) have suggested that the prostate is actually the source of seminal zymogens. Seminal fluid contains only Pg 6 and 7 (Seijffers et al 1965; Samloff & Liebman 1972). The ratio of glutamate: aspartate (including amides) and leucine: isoleucine in seminal proteinase is similar to that of gastricsin (the enzyme derived from Pg 6 or Pg 7) (Ruenwongsa & Chulavatnol 1977). Samloff and Townes (1970 a) showed that urine normally contains Pg 1-5. However there was a definite, although infrequent, finding of Pg 6 and 7 in urine from healthy subjects. Thus, although there was no direct evidence, the/....

the authors suggested that Pg 6 and 7 may normally be bound to serum proteins, preventing excretion, unless all such binding sites are saturated. On the basis of these differences in localization, pepsinogens 1-5 were named Pg I and 6 and 7 were named Pg II.

Etherington and Taylor (1970) showed that fundus produces both 'pepsin' and'gastricsin', while the pylorus produces only 'gastricsin', as judged by chromatography on Amberlite IRC 50. It was reported that pepsin 5 from pylorus had different electrophoretic mobility from pepsin 5 from fundus, whose mobility was the same as that of pepsin 5 from gastric juice; it was suggested that pyloric pepsin 5 might not be secreted into gastric juice. It should be stressed that this conclusion was reached solely on the basis of electrophoretic mobility.

The situation was considerably clarified by Samloff and Liebmann (1973) who raised antibodies in rabbits to PG I (from urine) and Pg II (from seminal fluid); these were labelled with fluorescein. Using anti-Pg II, specific fluorescence of chief and mucous neck cells was found, the fluorescent material being evenly distributed throughout the cytoplasm in densely-packed granules. Pyloric glands and Brünner's glands were similarly stained. Parietal cells were not stained. Zymogens of Pg I were located in chief and mucous neck cells but not in the pylorus or duodenum. Samloff (1971 a) pointed out that the mucous neck cell is probably the precursor of the chief cell, which could explain the similarity of their reaction. It should be noted that in the fundus the same cells appear to produce both Pg I and Pg II.

1.3.3 Control of Pepsinogen Secretion

This has been reviewed by Hirschowitz (1967) and Samloff (1971 b). The role of the vagus nerve seems to be central to the system. The vagus can be stimulated by the sight, smell and taste of food. The work of Wright et al (1975) indicates that secretion of the most electronegative of the cat pepsinogens is especially sensitive to vagal stimulation.

There are a number of gastrointestinal hormones that play a role in control of pepsinogen secretion. Of these gastrin is probably the most important. Pentagastrin, which is frequently used to stimulate gastric secretion in clinical trials, consists of the five C-terminal amino acids of gastrin (Samloff 1971 b).

1.4 Immunochemistry

Seastone and Herriott (1937) found that antisera raised against pig pepsin would not react equally well with porcine or bovine pepsins. Anti-pig pepsin reacted with pig pepsinogen and pepsin, but anti-pepsinogen would react only with pepsinogen. The authors pointed out that, although at the pH of body fluids pepsinogen would be native the pepsin may actually be denatured, so some of their data might be misleading.

Rapp et al (1964) showed that, of their four potentially-active fractions obtained from noncancerous human gastric mucosa, P II, III and IV were immunochemically different from each other.

Kushner et al (1964) showed that the human pepsinogen P II exhibited considerable cross-reactivity with pepsinogen A, while P I did not, although this may have been due to low concentration. The zymogens and their acidified counterparts showed considerable/....

considerable cross-reactivity. It was found that P IV was immunologically unrelated to the others.

Samloff (1971 c) studied the antigenic relationships of the human pepsinogen groups Pg I and Pg II. The source of immunizing antigen was urine which, after purification, contained only Pg 2-4 (members of Pg I). Antibody was raised in rabbits, and anti-Pg 2-4 Ig G was obtained. Double diffusion, immunoelectrophoresis and immunoabsorption studies indicated that anti-Pg 2-4 would react with extracts of human gastric mucosa and with urine (containing only Pg I), but not with extracts of duodenal mucosa (containing only Pg II) or with SMP. Anti-Pg 2-4 also reacted with Pg 1 and 5, both members of Pg I.

Samloff and Liebmann (1972) found that rabbit antiserum to purified seminal Pg II would react with seminal fluid and duodenal mucosal extracts. Anti-Pg II would not react with urine nor did anti-Pg I react with seminal pepsinogens.

It is thus clear that the original division of human pepsinogens into groups Pg I and Pg II can be justified on the basis of their immunochemical differences.

1.5 Genetics of Pepsinogens

Ogita (1968) proposed the classification of isoenzymes into unigenic and multigenic isoenzymes. Members of a unigenic set have distinct molecular characteristics, but are all synthesized under the control of one gene. Their differences in molecular structure must arise by post-synthetic modifications, e.g. substitution by small radicals, conformational changes, polymerisation etc...

etc. Enzymes within a unigenic set are immunochemically indistinguisable. Multigenic isozymes are controlled by two or more genes. There are two types of multigenic isozymes, allelic and non-allelic. Allelic isozymes are controlled by codominant allelic genes, while nonallelic isozymes are controlled by genes at different loci. Enzymes within an allelic set are generally immunochemically indistinguishable; those within non-allelic sets can be distinguished immunochemically.

Hanley et al (1966), in a study of electrophoretic mobilities of pepsinogen from several species, suggested that several zymogens of any one species are probably the products of a single gene. If so, any molecular differences would be due to post-synthetic modifications.

Samloff and Townes (1970 a), investigating the occurrence of group I pepsinogens in urine, found that individuals who lack Pg 5 in gastric mucosa do not secrete this zymogen in urine. Two patterns of excretion were defined, Pg^a (excretion of Pg 5) and Pg^b (absence of Pg 5). A study of seventy-five unrelated families led to the conclusion that absence of Pg 5 is inherited as a simple, autosomal recessive trait.

Further work by Samloff and Townes (1970 b) showed that, in 931 unrelated Caucasians (405 males), without known ulcer, 86% were of the A phenotype. The possible genetic determination of the patterns was studied in 100 matings involving seventy-five unrelated families, and it was confirmed that Pg 5 is controlled by/....

by allelic genes Pg^a and Pg^b, with Pg^a dominant.

Bowen et al (1972) described a further allele, Pg^c. Of 424 schoolgirls, 6% excreted predominantly Pg 4 in their urine, with most or all of the remaining activity in Pg 3. Patterns were consistent with codominance of Pg^c with Pg^a and Pg^b. It was concluded that there must be at least three alleles at the Pg locus.

A study of 499 Negroes by Townes and White (1974) uncovered 23 individuals who secreted neither Pg 4 nor Pg 5. This phenotype was designated B′. It was assumed that $Pg^{b'}$ is recessive to Pg^a and Pg^b.

Weitkamp and Townes (1975) and Weitkamp et al (1975) tried to test the hypothesis that all group I pepsinogens are determined at one locus. They found eighteen different patterns among 159 unrelated individuals, and showed that control of intensity of any one isozyme is probably related to that of the others. Their evidence from linkage analysis was insufficient to conclude that there is more than one locus, although the linkage of Pg 2 and Pg 4 with the HLA locus was considerably weaker than that of Pg 5. The data were consistent with the hypothesis of one locus but did not constitute definite proof. The authors realised that difficulty in classifying some patterns justified great caution in interpretation.

Weitkamp (1978) further studied the allelism of genes controlling intensity of Pg 4 and presence of absence of Pg 5. The data were somewhat ambiguous, and it was suggested that they fit the hypothesis of such allelism but do not establish it. It was/...

was also suggested that a better way of pepsinogen phenotyping would be useful.

A total of nine phenotypes was identified by Taggart et al (1979), differentiating between intense, faint and absent electrophoretic zones for Pg 2-5. An intense Pg 5 was thought to result from an allele Pg^d at the same locus as Pg^a, Pg^b and Pg^c. It was also proposed that the $Pg^{b'}$ allele postulated by Townes and White (1974) is artefactual, arising from low concentration, as opposed to absence, of Pg 4. Also Taggart and his coworkers, by evaluating the intensity of all pepsinogens jointly, found that heterozygous phenotypes, e.g. AB, show some characteristics of both AA and BB, thus indicating codominance. This contrasts with the work of Samloff and Townes (1970b) who, examining the intensity variations separately, proposed that Pg^a is dominant to Pg^b. Linkage analysis by Taggart et al excluded close linkage of the Pg locus with chromosomes 6 or 20. Chromosome 6 is the site of the HLA locus.

Whittington et al (1980) performed linkage studies for glyoxylase, Pg, HLA and spinocerebellar ataxia. Although there was not a great deal of data for Pg, there was no evidence of linkage of Pg to HLA.

This disagreement concerning the linkage or otherwise of the Pg locus with chromosome 6 has yet to be resolved.

Korsnes and Gedde-Dahl (1980), in describing 37 phenotypes, provided some evidence that Pg 2,3,4 are not coded by the Pg 5 locus. Further, they suggested that one of the loci, Pg 5, is polymorphic with 4 known alleles. Firm conclusions about Pg 2, 3 or 4 could not be made although it was postulated that Pg 2 is dependent/....

dependent on, and possibly a secondary product of, Pg 3. The authors felt they could not ignore the possibility that the group I pepsinogens are not in one cluster, although this was not a definite conclusion. There was considerable difficulty in scoring the intensity of at least one band, so it may be that some of the phenotypes are suspect.

All of these studies rely on the subjective assessment of absence or presence of electrophoretic zones and of the relative intensities of those bands which are present. Thus, it is frequently difficult to distinguish between neighbouring zones, as discussed by Weitkamp and Townes (1975). Also, variations in amounts of pepsinogen excreted in urine could be physiologically mediated, e.g. Taggart et al (1978) give details of one individual who on three occasions exhibited one phenotype, and on a fourth evaluation was found to have a different phenotype. Physiologically increased or decreased pepsinogen levels could explain this situation. It should be noted that little is known about postsynthetic modification. Such modifications (e.g. addition of phosphate or carbohydrate) are know to occur in some pepsins (Lee & Ryle 1967: Kageyama & Takahashi 1977). Further biochemical analyses of human pepsinogens, especially amino acid analysis, would help solve the problem of whether or not Pg 2 - 5 are determined at one gene.

Although there appears to have been no work done on the genetics of group II pepsinogens it is probable that the known differences between groups I and II in antigenic determinants, distribution in body fluids and enzymatic properties of the pepsins result from/.....

from the expression of different loci.

1.6 Pepsins and Ulceration

Peptic ulcer is not a single disease. The most common sites for this lesion are the lesser curvature of the body of the stomach and the duodenum proximal to the exit from the stomach. Peptic ulcer may also occur in the oesophagus, jejunum and ileum.

A great deal of clinical work has been done on the aetiology of ulceration. Both acid and pepsin appear to be necessary, although it is not clear if both play a positive role or whether the acid merely provides the environment in which pepsin can work. A number of studies have shown that about one third of duodenal ulcer patients secrete increased acid and pepsin, while the remainder, along with gastric ulcer patients, secrete acid and pepsin within the normal range (Janowitz & Hollander 1952; Van Goidsenhoven et al 1958; Grossman 1976). It has been suggested that the alkaline secretions of the duodenum may denature pepsins and thus protect against ulceration. However, the pepsin secreted by the duodenum (gastricsin, group II pepsinogen) has increased alkali-stability (Seijffers et al, 1964 b). Furthermore, duodenal pH may be as low as pH 3 for considerable lengths of time (Aynaciyan & Bingham 1969). Also the buffering effect of food proteins may be lost in duodenal ulcer patients, who empty the stomach more rapidly than normal (Grossman 1976).

Many workers regard ulceration as the result of inbalance between acid and pepsin secretion and mucosal resistance, although the nature of this resistance is unclear. It is known that acid freely/....

freely diffuses through the mucous layer (for review, see Glass 1976). It has been suggested that bile refluxing from the duodenum may break the gastric "mucosal barrier" and thus contribute to ulceration (Grossman 1976). An in vitro study has indicated that bile salts may actually inhibit pepsin activity (Tompkins & Croke 1980). These workers used commercial pig pepsin, and it may be that this effect is not relevant to human physiological conditions. Pearson et al (1979) have shown that pepsin will degrade the surface mucous gel of the pig stomach by destruction of the polymeric structure of its glycoprotein. The authors did not present any data on the differences, or otherwise, between ulcerated and healthy stomachs. They suggested that changes in the integrity of the mucous gel may alter its protective effect.

Taylor (1970), using agar gel electrophoresis, assessed the secretion of pepsins by a range of human subjects. A semi-quantitative estimation of each zone was made by eye, ranging from 0 for no activity to 4 for maximal activity. Pepsin 1 was present in 47% of non-ulcerated subjects, 68% of duodenal ulcer patients and 78% with gastric ulcer. The difference between the frequencies in normal and gastric ulcer subjects was significant, that between normal and duodenal ulcer subjects was not. It was concluded that occurrence and amount of the most electronegative pepsin, pepsin 1, was related to gastric and duodenal ulceration. However, the method appears to be rather subjective.

Whitecross et al (1974) used agar gel electrophoresis, but with considerably high potential gradient and longer duration of electrophoresis/....

electrophoresis than Taylor (Etherington & Taylor 1969). A somewhat different pattern was observed from that of Taylor, consisting of either 4 or 5 bands depending on the pH of gastric juice when collected. A total of 48 subjects was examined, 11 being controls; no change in pattern was observed in subjects with gastric or duodenal disease compared to normals. However, these authors did not estimate the intensity of individual bands. Whitecross et al also correlated electrophoretic patterns with chromatographic patterns using DEAE-cellulose, and eluting pepsins with a salt gradient up to 0·23 M NaCl. Thus, the major pepsin present was shown to be one of the fast-moving bands. However, the work of Taylor and his colleagues has shown that a gradient up to 0·8 M NaCl is needed to remove minor pepsins 1 and 2. It seems likely that Whitecross et al failed to remove pepsins 1 and 2 from their columns, and also that they electrophoresed these pepsins off their gels.

Attempts have been made to establish a differential assay whereby individual pepsins in gastric juice can be estimated without fractionation. Such an assay might be useful in studies of the role of pepsins in ulceration. Chiang et al (1966) used APD and haemoglobin in this way, on the basis that only pepsin (not gastricsin) will hydrolyse A.P.D., while both enzymes will hydrolyse haemoglobin.

Auffret and Ryle (1979) synthesised a number of peptides of the type A-Leu-Val-His-B, to study the catalytic activity of pig pepsin C. Substrates in which A=Ac-Tyr are poor substrates for pig/...

pig pepsin A. Ryle & Auffret (1979) suggested that Ac.Y.L.V.H. NH_2 might replace haemoglobin in the differential assay. The use of Ac.Y.L.V.H.NH_2 would allow assay of gastricsin, while A.P.D. would allow assay of pepsin. Cross-reactivity of gastricsin against A.P.D. and pepsin against Ac.Y.L.V.H.NH_2 could be taken into account by the use of simultaneous equations, as constructed by Auffret (1977). It was suggested that such an assay system is easy to perform, reliable and might be automated. Complications may arise if more that two different enzymes are present.

This project represents an attempt to assess the feasibility of this approach.

2.1 Collection of gastric juice

Samples of gastric juice were collected by hospital staff from patients undergoing acid output tests and from volunteers who had given their informed consent at the Royal Infirmary and the Western General Hospital, Edinburgh.

After overnight fasting, the basal secretion was collected over a period of one hour. Pentagastrin (6μg/kg body weight) was administered intravenously and the stimulated secretion was collected for a further hour. Unless otherwise stated, the total secretion was pooled.

The gastric juice was centrifuged in a MSE 18 centrifuge at 20,000 g for 20 minutes, at 4° C, to remove mucus. The pH of the supernatant was measured on a Corning-Eel Model 12 pH meter. The gastric juice was dialysed against at least 10 volumes of distilled water, with three changes of water. A brown precipitate, possibly due to bile pigments, which invariably formed was removed by centrifugation, as above. The dialysed gastric juice was then freeze-dried. Assay of the sample by haemoglobin digestion (Sect. 2.2) before and after freeze-drying showed that loss of activity due to this process did not exceed 10%. Freeze-dried material was either stored at 4° C or used immediately.

2.2. Determination of Enzymic Activity using Bovine Haemoglobin as Substrate

The method used for the assay of human pepsins was that of Tang (1970) with slight modification.

Bovine haemoglobin enzyme substrate powder was obtained from Armour Pharmaceuticals Ltd., Eastbourne, England. A stock 10% (w/v) solution in distilled water was prepared, and this was centrifuged in a MSE 18 centrifuge at 20,000 g for 15 minutes, at 4° C, to remove insoluble material, the solution being too thick to filter. The centrifuged solution was dialysed against three changes of distilled water, to remove low molecular weight material, whose presence would present unacceptably high blank values. Thiomersal (2·5 mg/ 100 ml) was added to this solution as a preservative, and it was stored at 4° C. The solution was viable for several weeks.

Haemoglobin stock solution was acidified just before use by the addition of 6 ml of 0·3 M HCl to 20 ml of haemoglobin. This was made up to 100 ml with distilled water, and the pH was checked. If necessary it was adjusted to pH 3·1, using NaOH or HCl. This solution was incubated at 37° C for about five minutes, to allow complete denaturation of haemoglobin.

Enzyme solution (0·25 ml) in water, or in the buffers used for chromatography, and 0·25 ml of 0·1 M citrate buffer, pH 3·1, were incubated together at 37° C for several minutes. Acid haemoglobin (2·5 ml) was added, the reagents were shaken manually for 3 - 4 seconds, and the reaction was allowed to proceed at 37° C for 10 minutes. The final pH of the incubation mixture, pH 3·1, was not affected if the enzyme was originally present in chromatography buffers. The reaction was stopped by the addition of 5 ml of 5% (w/v) trichloroacetic acid (TCA) solution. The reagents were mixed in a Whirlimixer, and allowed to stand for at least 10 minutes to ensure complete precipitation of protein. The mixture was filtered through Whatman No. 3 filter/...

No. 3 filter paper, and the absorbancies of the filtrates were read at 280 nm in a Unicam SP 500 spectrophotometer. Blank values were obtained by adding TCA solution before haemoglobin. Duplicates of "live" and blank assays were performed.

The difference in absorbance between the mean "live" samples and the blanks (i.e. ΔA_{280}) was calculated. Specific activities are expressed as $\frac{\Delta A_{280} / \text{ml of enzyme sample}}{A_{280} \text{ of sample}}$

The linearity of this assay for some pepsins is shown in Sect.4.1.1.

2.3 Determination of Enzymic Activity using Acetyl-L-Phenylalanyl-L-di-iodotyrosine (A.P.D.) as substrate

In initial experiments, the methods of Tang (1970), slightly modified, and of Ryle and Porter (1959) were compared. It was found that the modified Tang method was more sensitive for the assay of the major human pepsin. Thus in all subsequent work, this method was used; modification took account of the need to adjust buffered enzyme samples to the appropriate pH by using suitable concentrations of HCl for acidification. The A.P.D. was obtained from Yeda, Rehovoth, Israel.

A stock solution of 1 mM A.P.D. in 5 mM NaCH was prepared and stored at 4°C. The substrate must first be dissolved in 0·1 M NaOH thendiluted with distilled water. Ninhydrin reagent was the modified reagent of Moore and Stein (1954); it was stored in a dark glass dispenser under nitrogen. For routine assays,the final pH was pH 2.3.

All assays, including blanks, were performed in duplicate. Enzyme solution (0·5 ml), in distilled water or buffer, and 0·25 ml of HCl of the appropriate concentration were incubated together at 37° C for several/

several minutes.

A.P.D. solution (0·25 ml), previously equilibrated to 37° C, was added to the enzyme/HCl mixtures. The tubes were shaken manually, and the reaction was allowed to proceed for one hour. At the end of this time, 1 ml of ninhydrin reagent was added, and the tubes were again shaken manually. They were then placed in a boiling water-bath for 15 minutes. After the tubes had been cooled in water, 5 ml of 60% (v/v) aqueous ethanol were added. The reagents were thoroughly mixed by vortexing, and the absorbances at 570 nm of the contents were measured in a Unicam SP 500 spectrophotometer. Blanks were set up by adding ninhydrin before A.P.D. Specific activities are expressed as

$$\frac{\Delta A_{570}/\text{ml of enzyme sample}}{A_{280} \text{ of sample}}$$

The linearity of the assay is shown in Sect. 4.1.2.

2.4 Determination of Enzymic Activity using Acetyl-L-Tyrosyl-L-Leucyl-L-Valyl-L-Histidyl amide (Ac.Y.L.V.H.NH_2) as substrate

The substrate was prepared in the laboratory by Dr. A.P. Ryle. A stock solution of 0·5 mM substrate in citric acid/NaCH buffer of the appropriate pH was prepared and stored at 4° C. Buffers were 0·05 M in citrate ions. After the pH - optimum studies reported in Sect. 4.2, all further routine assays using Ac.Y.L.V.H.NH_2 were performed at pH 3·8.

Enzyme solution (0·5 ml) was equilibrated to 37° C, as was the substrate solution, 0·5 ml of which were added to the enzyme. The tubes were shaken manually and the reaction was allowed to proceed for 20 minutes. After this incubation, 1 ml of ninhydrin reagent was added and the /....

and the tubes were shaken. The tubes were placed in boiling water-bath for 15 minutes, after which they were cooled in water, and 5 ml of 60% (v/v) aqueous ethanol were added. The reagents were mixed by vortexing, and the absorbances at 570 mm were measured in a Unicam SP 500 spectrophotometer. Blanks were set up by adding ninhydrin before substrate. Specific activities are expressed as:

$$\frac{\Delta A_{570}/\text{ml of enzyme sample}}{A_{280} \text{ of sample}}$$

The linearity of the assay is shown in Sect. 4.1.3.

2.5 Determination of Enzymic Activity by the milk-clotting assay

The method used was essentially that of McPhie (1976). Dried skimmed milk powder was Gaylord-Hauser brand obtained from local pharmacists.

Milk powder was made as a solution of 9·5 g/100 ml. Milk solution (1 ml) plus 4 ml 0·1M $CaCl_2$ were mixed and made up to 50 ml with 0.2M acetate buffer pH 5·3. The absorbance at 500 nm of this solution was measured in a Unicam SP 500 spectrophotometer, thermostated at 25° C. The test solution was added and any change in absorbance was noted.

2.6 Purification of Proteins by Ion-Exchange Chromatography

For all chromatography, buffers were prepared from acids and their sodium salts, of Analar grade obtained from BDH Chemicals Ltd., Poole, England. Concentrations of the buffers given are the concentrations of their anionic components.

The media used for ion-exchange chromatography were:-

(a) Amberlite IRC 50 (CG 50) from BDH Chemicals Ltd., Poole, England.

(b) DEAE- cellulose, in the microgranular DE-52 preswollen grade, from Whatman Labsales Ltd., Maidstone, Kent, England.

(c)/...

(c) DEAE-Sephadex A50 from Pharmacia Ltd., Uppsala, Sweden.

(d) DEAE-Sephadex A50 from Sigma Chemical Corp., St. Louis, Mo., U.S.A.

The two batches of DEAE-Sephadex A50 gave identical results.

In all cases, the resin was pre-washed, if necessary, according to manufacturers' instructions, and "fines" were removed. It was found that equilibration of the material with the appropriate buffer was made easier by stirring the exchanger in a solution of buffer which was twenty-fold more concentrated than that used during chromatography. When the correct pH was reached, the resin was washed four or five times with buffer of the correct ionic strength, the resin was de-gassed at the water pump, the column was poured, and the resin was allowed to settle under gravity.

These preparatory steps were always performed at room temperature and the columns were transferred, where appropriate, to the cold room, at 4° C. A volume of starting buffer, usually about 200 ml, was run through the column to ensure equilibration.

The sample, either freeze-dried gastric juice or partially-purified protein, dissolved in starting buffer, was centrifuged in a MSE 18 centrifuge at 16,000 g for 15 minutes at 4° C. An insoluble pellet, possibly consisting of denatured protein, which formed was washed with starting buffer and re-centrifuged under the above conditions, and the supernatants were pooled. If freeze-dried gastric juice was being used it was preferable that its concentration should not exceed 10% (w/v), otherwise it tended not to permeate the resin bed.

In the case of DEAE - cellulose and DEAE - Sephadex A50 it was found that the pooled, freeze-dried gastric juice of five/....

five subjects could be applied at any one time for preparative purposes. Any more than this caused overloading and consequent lack of resolution of peaks.

Total protein (as measured by absorbance at 280 nm) and haemoglobin - digesting activity were measured, so that recoveries could be calculated.

2.6.1 Chromatography on Amberlite IRC 50

When using Amberlite, the chromatogram was developed by step-wise increases in pH similar to the method given by Tang (1970).

In the work reported here, 0·2 M citrate buffers of pH 3·0, 3·4, 3·8, 4·2 and 4·6 were used. The pH 3·4 buffer was not used by Tang, but was used here as an extra wash which removed a small amount of enzymically-inactive material. Chromatography was performed at room temperature. The flow rate was maintained at 80 ml/hr, using a LKB Type 10200-1 Peristaltic Pump. Buffer was applied to the column under positive pressure. Fractions of approximately 25 ml were collected using a Central Fraction Collector.

2.6.2 Chromatography on anion exchangers

Chromatograms using DEAE-cellulose and DEAE-Sephadex A 50 were developed using gradients of sodium chloride concentration, with buffers of constant pH.

Following sample application, the columns were initially washed with buffer free from NaCl, until no further protein was eluted, as determined by the absorbance of fractions at 280 nm. Fractions of approximately 5 ml were collected throughout using a Central Fraction Collector. A flow rate of 20 ml/hr was maintained using the LKB Peristaltic Pump, buffer being applied under positive pressure.

The chromatograms were further developed by exponential gradients of NaCl concentration. The first gradient used was produced by passing buffer similar to the starting buffer, but containing 0·2 M NaCl, from an open reservoir into a closed mixing vessel containing 100 ml of starting buffer. The contents of the mixing vessel were continuously stirred by a magnetic stirrer.

When no further protein was eluted and the concentration of NaCl in the eluant had nearly reached 0·2 M, the buffer in the reservoir was replaced by buffer containing 1 M NaCl and this gradient was applied.

On various occasions different gradients of NaCl were used, but the method of production of the gradients remained the same.

It was found the DEAE - Sephadex A50 shrank markedly during application of the buffer containing 1M NaCl, to a final height of about 4 - 5 cm, from an initial height of 17 cm. Both DEAE - cellulose and DEAE - Sephadex were regenerated by washing with starting buffer in situ, until the material had re-swollen and there was no trace of NaCl in the eluant. Shrinkage of DEAE-cellulose was comparatively slight.

The chloride ion content of chromatographic fractions was determined using an EEL chloride meter.

2.7 Purification of Proteins by Gel Filtration

The preparation and pouring of the columns was performed at room temperature (except where stated), and the columns were run at 4° C.

Sephadex G 75, obtained from Pharmacia Ltd., Uppsala, Sweden, was swollen in 0·1 M acetate buffer, pH 4·0, by placing sufficient gel, in excess buffer, in a boiling water bath for three hours or in excess/

excess buffer at room temperature for 24 hours. Columns were 2·5 x 40 cm. Following swelling of the resin, the material was degassed and columns were poured and allowed to settle under gravity, then carefully checked for imperfections.

Partially-purified proteins were dissolved in 0·1 M acetate buffer, pH 4·0. It should be noted that, for gel filtration, it is important that the sample should be dissolved in a relatively small volume in order to obtain sharp resolution of peaks. Thus, the volume of sample used was never more than 3 ml. The samples were centrifuged in a MSE 18 centrifuge at 16,000 g for 15 minutes at 4°C. The supernatants were applied to columns of Sephadex G75, the surface of the columns being protected by filter paper to prevent disturbance of the resin.

The proteins were eluted by washing the columns with 0·1 M acetate buffer, pH 4·0. Flow rate was maintained at 20 ml/hr using the LKB Peristaltic Pump, buffer being applied under positive pressure. Fractions of 5 ml were collected using a Central Fraction Collector.

2.8 Electrophoresis of Proteins

2.8.1 Electrophoresis using Vertical Polyacrylamide slab gels.

Acrylamide, specially purified for electrophoresis, polyacrylamide (batch 6560570) and ammonium persulphate were obtained from BDH Chemicals, Poole, England. TEMED and BIS were obtained from Koch-Light Laboratories Ltd., Colnbrook, England.

The electrophoresis unit was made in the Workshop at the Dept. of Biochemistry, Edinburgh. It was similar to that available commercially from Raven Ltd., Haverhill, Suffolk, England.

Stock acrylamide solution was 30% (w/v) acrylamide and 0·8% (w/v) BIS in/.....

BIS in water. This was stored at 4° C. Ammonium persulphate solution (20 mg/ml) was made fresh each day; TEMED was used undiluted. Stock polyacrylamide solution was 30% (w/v) in water: it was stored at room temperature with 0·01% sodium azide as preservative. Inclusion of polyacrylamide gave extra strength to gels so they could be more conveniently handled. Experiments in which polyacrylamide was omitted indicated that this material did not affect electrophoretic mobilities.

Glass plates were used, one being 20 x 16 cm the other 17 x 16 cm, both 3 mm thick. Teflon strips sealed with high-vacuum grease, were positioned down the sides to separate the plates. The cassette was held together by Bulldog clips.

For some gels, the general approach used was that of Williams and Reisfeld (1964) in which a stacking gel of different pH from that of the separating gel is incorporated. This is intended to produce concentration of relatively dilute samples with consequent band sharpening. In these gels, chloride ion was used as the leading ion with acetate ion as the trailing ion, and β-alanine as the buffer component. Gel buffer solutions were prepared by titration of 100 ml of 1M β-alanine with HCl to the appropriate pH and dilution to 200 ml with water. Three distinct gels were used as outlined below.

The supporting gel solution consisted of 13 ml stock acrylamide, 5 ml β-alanine/HCl buffer pH 4·5 and 2 ml ammonium persulphate, to which 40 µl of TEMED were added. The gel cassette was held vertical in an aluminium foil trough, and the gel was poured to a depth of 2 cm and allowed to set, after which the cassette was removed from the trough.

The/

The separating gel was then cast to a depth of about 12-13 cm. The gel solution consisted of 8 ml acrylamide, 7·5 ml β-alanine/HCl buffer pH 4·5, 2 ml ammonium persulphate, 5 ml polyacrylamide, 7·5 ml water, to which 30 µl TEMED were added after degassing. After pouring, the gel was covered by a layer of water-saturated secondary butanol, to produce a smooth surface. The butanol was washed off when the gel had set.

At this point, it was possible to store the gel overnight. The gel was layered with buffer of the same pH and concentration as that in the gel. The whole cassette was wrapped in "Cling Film" and stored at 4° C.

Alternatively, the final gel could be cast immediately.

In cases where the separating gel had been stored overnight, it was often found that the stacking gel would not set properly on top of the separating gel, because of the presence of dissolved oxygen which inhibits polymerization of acrylamide. Thus, after removal of the buffer under which the separating gel had been stored, a solution of 10% (w/v) sodium dithionite in water was applied to the top of the gel. This was allowed to stand for five minutes and rinsed off with distilled water.

The stacking gel solution consisted of 1·33 ml acrylamide, 1·66 ml β-alanine/HCl buffer pH 3·5, 1·5 ml ammonium persulphate, 1·66 ml polyacrylamide and 3·85 ml water; after this was degassed, 40 µl of TEMED were added. The top of the separating gel was rinsed with this solution, the gel was poured, and the slot former was inserted. When the gel had set the slot former was removed, and the cassette was placed in position secured to the electrophoresis unit by Vaseline and Bulldog clips.

β-alanine/acetic acid buffer pH 3·6 (0·05 M in β-alanine) was placed in the upper and lower chambers.

Samples were made up as solutions of 1 mg/ml in water, and centrifuged in a MSE bench centrifuge. In many cases, a small precipitate was removed. Failure to centrifuge samples produced streaky gels due to the presence of insoluble material. A few drops of 10% (w/v) sucrose plus 0·02 % (w/v) bromophenol blue, in water, were added to each sample. Samples (10-50µl) were applied to the gel, and electrophoresis was performed at constant voltage, 150 v, for about four hours. The initial current was usually about 30 mA; this decreased during electrophoresis.

The gels were stained for activity by a process based on that of Uriel (1960).

In an attempt to minimise diffusion through the gels, and consequent band broadening, the staining time was reduced. Thus, at the end of the electrophoresis, the plates were carefully separated with a spatula and the gels were placed in a suitable dish. Removal of the gel from the plates was made easier by directing a trickle of water between gel and plate.

The gels were soaked in a 4:1 mixture of 2·5% (w/v) haemoglobin solution and 0·39 M HCl at room temperature for 30 minutes. They were transferred to a solution of 20% (v/v) methanol plus 10% (v/v) acetic acid in water, and fixed for 10 minutes at room temperature. Subsequent staining of the gel background was improved by the use of Coomassie brilliant blue R instead of amido black. The dye solution was made up by dissolving 1·25 g of Coomassie blue in 227 ml of methanol, to which was added 46 ml of glacial acetic acid; the volume was made up to 500 ml with water. The dye was filtered through Whatman/....

Whatman No 54 filter paper. After fixing, gels were stained in this dye for 5 minutes at 37° C.

Gels were destained in a solution of 5% (v/v) methanol plus 7·5% (v/v) glacial acetic acid, in water. Destaining was facilitated if the gel dishes were placed in a shaking water bath at 37° C overnight. Pieces of wool placed in the destaining solution helped mop up the dye. After destaining, areas of enzymic activity appeared as white bands against a dark background, representing areas in which haemoglobin had been digested.

In other experiments the stacking gel was omitted. All stock solutions were as described earlier, except the gel buffer which was 0·1 M acetate, pH 4·0. The supporting gel was 20% acrylamide, 25 mM acetate buffer, pH 4·0. The separating gel was the only other gel used. This gel solution consisted of 10 ml stock acrylamide, 10 ml 0·1 M acetate buffer pH 4·0, 7 ml polyacrylamide, 2·6 ml ammonium persulphate plus 10 ml water. This was degassed and 40 µl of TEMED were added. Electrophoresis was performed and gels were stained as described above.

Molecular weights of proteins were determined using polyacrylamide gel electrophoresis by a modification of the method of Laemmli (1970). The electrophoresis unit, stock solutions of acrylamide, polyacrylamide and ammonium persulphate, and TEMED were the same as those described above. Stock SDS solution was 10% (w/v) in water. All gel solutions were degassed before addition of TEMED.

The supporting gel consisted of 16·7 ml acrylamide, 4·6 ml of 2M Tris/HCl buffer pH 8·8, 0·34 ml SDS, 2·4 ml ammonium persulphate plus 20 µl TEMED.

The separating gel contained 16·5 ml acrylamide, 13 ml 1 M Tris/HCl/....

Tris/HCl buffer pH 8·8 containing 5·35 mM disodium EDTA, 0·35 ml SDS, 4 ml polyacrylamide, 1 ml ammonium persulphate plus 20 µl TEMED.

The stacking gel contained 1·33 ml acrylamide, 1·4 ml 1 M Tris/HCl buffer pH 6·8 containing 16 mM disodium EDTA, 0·11 ml SDS, 0·5 ml ammonium persulphate, 6·7 ml water plus 10 µl TEMED.

The stock electrode chamber buffer was 0·25 M Tris/HCl buffer, pH 8·3, containing 1·92 M glycine, 1% (w/v) SDS and 20 mM disodium EDTA. This was diluted tenfold before use.

The stock dissociation buffer consisted of 40 ml 0·5 M Tris/HCl buffer pH 6·8, 8 g SDS, 40 ml glycerol, 4 ml 0·2 M disodium EDTA, pH 7·0, water up to 100 ml.

Protein samples (freeze-dried chromatographic fractions and molecular weight standards) were made up as 4 mg/ml solutions in water and centrifuged in a MSE bench centrifuge. A mixture of 50 µl sample, 25 µl dissociation buffer, 12 µl 50% mercaptoethanol, and 13 µl 0·02% (w/v) aqueous bromophenol blue was placed in a boiling water-bath for 2 minutes. Samples were then applied to the gel. Electrophoresis was conducted at constant voltage, 50 V, overnight, after which the gels were fixed, stained in Coomassie brilliant blue and destained as described earlier.

2.8.2 Agar gel electrophoresis

The method of Etherington and Taylor (1969) was used with slight modification. Glass plates, 20 x 10 cm, were covered with a 1·5 % (w/v) solution of agar (Ionagar No. 2, Oxoid Ltd., London EC4) in 25 mM acetate buffer, pH 5·0. Pepsins were prepared as 0·1 mg/ml solutions/....

solutions in 1 mM HCl, except for swine pepsin which was 0·02 mg/ml. The samples were mixed with 3% agar at 50° C and pipetted into 10 x 1 mm slots cut 5 cm from the cathode end of the agar plates. The buffer in the electrophoresis tanks was 0·1 M acetate buffer, pH 5·0. Wicks made from Whatman 3 MM paper connected the tanks with the gels which were covered with polythene to prevent evaporation. Electrophoresis was performed at 10 V/cm for 3 hours at 4° C. The gels were then incubated at 37° C for 45 minutes in a 0·33% (w/v) solution of human globin, prepared by Dr. N. Roberts by the method of Ito et al (1964), in 0·2 M glycine/HCl buffer pH 2·0 containing 0·1 M NaCl. The globin was decanted and the gels were left at 37° C for a further hour, then fixed overnight in a solution of methanol: water (1:1) containing 7% acetic acid. The gels were stained in Ponceau S (1g in 500 ml 1M acetic acid plus 500 ml 0·1 M sodium acetate) for 1½ hours, destained in 7% acetic acid and dried on transparent film, dried at 60-70° C, after which negative photographs were obtained using a standard office copier (Dalcopier, W.A. Goddard, Manchester, U.K.).

2.9 Amino Acid analysis

2.9.1. Acid hydrolysis

Samples of freeze-dried protein of known weight (all about 0·5 mg) were placed in glass hydrolysis tubes previously washed in persulphuric acid. Fresh hydrolysis mixture (0·3 ml of 6 M HCl plus 10 µl of 1% (v/v) aqueous thioglycolic acid) was added to each sample. Thioglycolic acid prevents oxidation of methionine and tyrosine (Sletten et al 1968). The neck of each tube was drawn out to a narrow constriction in a flame, and the contents were frozen. The tubes were then evacuated and sealed under vacuum. Hydrolysis was conducted at 110° C for 20 hours, after which the tubes were opened and the contents were dried down in a vacuum desiccator in the presence of sodium hydroxide pellets.

Each sample was taken up in 0·5 ml of 0·1 M citrate buffer, pH 2·27, and 0·15 ml of this was applied to the amino acid analyser.

2.9.2 Performic acid oxidation

Cysteine and cystine are unstable during acid hydrolysis. These residues are therefore first oxidised to cysteic acid, which is stable during hydrolysis, and total half-cystine is estimated in this form.

Fresh performic acid was prepared by mixing 0·2 ml of 30% (v/v) hydrogen peroxide and 1·8 ml of 88% formic acid. These reagents were allowed to stand for one hour at room temperature. The performic acid was then cooled to 0° C in an ice bath, and 0·1 ml was added to a known weight (about 0·5 mg) of each freeze-dried protein. This mixture was left for a further hour in the ice bath, after which it was freeze-dried.

The samples were then hydrolysed in 6 M HCl as described above (Sect 2.9.1.). Because thioglycolic acid is used to prevent oxidation, it can obviously be omitted from this step. Hydrolysed samples were taken up in 0·1 M citrate buffer, pH 2·27.

2.9.3 Amino Acid Analyser

The analyser used was a Locarte floor model Mark IV with a 20 cm x 1cm sulphonated polystyrene resin ion-exchange column. The analyser was equipped with a 12-sample autoloader and a Spectra-Physics System Amino Acid "Autolab" integrator.

Samples prepared as described above were applied to the analyser and blown in with nitrogen, then overlayered with a few drops of the pH 2·27 buffer. The subsequent method was based on that given by Spackman et al (1958).

2.9.4 Reagents used in Amino Acid Analysis

The first buffer, which removes acidic amino acids, was 0·1 M

citrate buffer, pH 3·25, containing 0·12 M NaCl. Thiodiglycol (0·5% v/v, pH 3·25) was added as an anti-oxidant to protect methionine and Brij 35 (6·3 g per 9 dm^3) was added to improve the flow-rate of the column (Moore and Stein 1951). Pentachlorophenol (30 drops per 9 dm^3) was added as a bacteriocide.

The second buffer was 0·1 M citrate buffer, pH 4·25, containing 0·055 M NaCl, plus thiodiglycol (0·5% v/v, pH 4·25) and Brij 35(as above). This buffer removes neutral amino acids.

The final buffer used was 0·1 M citrate, 0·033 M borate, pH 9·35, which removes basic amino acids. The buffer contained 0·5 % (v/v) thiodiglycol, pH 9·35, and Brij 35 (as above).

The ninhydrin reagent was 2% (w/v) in ninhydrin, containing 0·04% stannous chloride, in a solvent consisting of 750 ml of methyl cellosolve plus 250 ml of 4 M acetate buffer, pH 5·5. The cellosolve was tested for the presence of peroxides by mixing equal volumes of 5% (w/v) potassium iodide and cellosolve; a deep yellow colour indicates the presence of peroxides, in which case the cellosolve is unusable. Methyl cellosolve may be purified by distillation, in the presence of 3·6 M H_2SO_4 (5-10 ml per dm^3) and a little $SnCl_2$; the cellosolve distils at 122° C.

2.9.5 Estimation of Amino Acids

All amino acids were detected at 570 nm, except proline which is not a primary amino acid and therefore reacts differently with ninhydrin; proline is detected at 440 nm.

A standard mixture of amino acids obtained from Sigma Chemical Co., containing all amino acids except cysteic acid and tryptophan, was applied to the analyser.

For the unknowns, amounts of all amino acids, except proline and cysteic/...

cysteic acid, were determined by the integrator. For the estimation of proline, the area of the proline peak on the standard chart was determined as the product of the peak height and the width of the peak at half-height. The colour constant was determined from the ratio of peak area: known amount of proline. For the unknowns, amounts of proline were determined from the ratio peak area: colour constant. For half-cystine (measured as cysteic acid) a similar calculation was performed using the colour constant for aspartate.

A computer program devised by Dr. A.P. Ryle was used to determine the number of residues per molecule for each protein, calculated from the estimated amino acid quantities and the molecular weight of the protein.

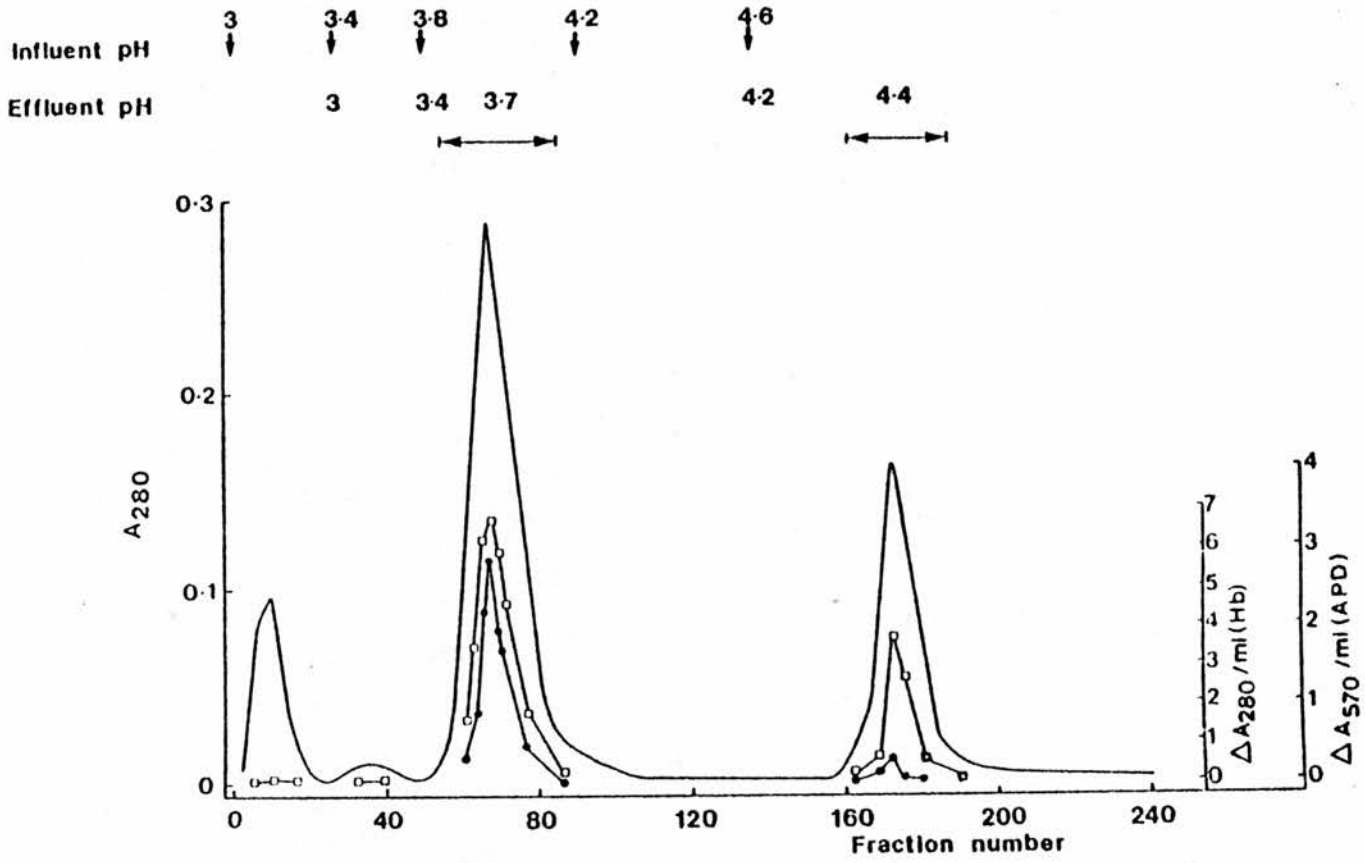

Fig. 1 Amberlite IRC 50 chromatography of human gastric juice, using stepwise changes of pH, 0·2 M citrate buffers. Column 4·5 cm x 25 cm; flow-rate 80ml/hr. Fractions (20 ml) were pooled as shown. — , protein; □, assay with haemoglobin (Hb); •, assay with A.P.D.

Chapter 3 PROTEIN PURIFICATION

3.1 Preparation of human pepsin and gastricsin on Amberlite IRC 50

Samples of human gastric juice were collected by staff at the Royal Infirmary and the Western General Hospital, Edinburgh and were prepared for chromatography as described in Sect. 2.1.

Freeze-dried gastric juice was fractionated by the method of Tang (1970) with slight modification, as detailed in Sect. 2.6. Full details of the chromatographic method are given in that Section. The sample (no more than 3 g) was dissolved in 0·2 M citrate buffer, pH 3·0, and was centrifuged and prepared for chromatography as described in Sect. 2.6. The sample was applied to a 4·5 x 25 cm column of Amberlite IRC 50, prepared as described in Sect 2.6, and equilibrated with 0·2 M citrate buffer, pH 3·0. The chromatogram was developed by stepwise increases in pH, at room temperature. The flow-rate was maintained at 80 ml/hr. Buffer was applied by positive pressure; details of changes of buffer are shown in Figure 1.

The absorbance at 280 nm of all fractions was measured in a Unicam SP 500 spectrophotometer. The enzymic activity of protein-containing fractions was determined by assay with haemoglobin and A.P.D. as detailed in Sects.2.2 and 2.3.

Fractions representing individual peaks of activity were pooled and dialysed against three changes of distilled water at 4° C. The fractions pooled are shown in Figure 1. The material was then freeze-dried and stored at 4° C.

The first/...

The first active peak ('pepsin') was eluted at pH 3·7 - 3·8, as was the pepsin peak in similar experiments performed by Etherington and Taylor (1969) and by Ryle and Auffret (1979); in contrast, the data presented by Tang (1970) show that the first peak was eluted at pH 4·0. The reason for this discrepancy is unknown, but the data reported here were reproducible. As may be seen from Figure 1, the extra wash at pH 3·4 removed a small amount of enzymically-inactive material.

Figure 1 shows that the second peak (gastricsin) was eluted from the column at pH 4·4. This agrees with the data of Tang (1970) and Etherington and Taylor (1969) but is slightly different from results presented by Ryle and Auffret (1979) whose gastricsin was eluted at pH 4·23.

For haemoglobin-digesting activity the specific activity of the pepsin was 23·1 and that of gastricsin was 23·8; these are considerably lower than the specific activities determined by Ryle and Auffret (1979), using the same assay conditions, as 39·5 and 85 respectively, although in this case the gastricsin had been further purified by chromatography on DEAE-cellulose. Such differences in specific activity might be explained by differences in handling gastric juice, such that in the work reported here increased loss of activity occurred during preparation of samples. Specific activities were calculated as in Sect. 2. With A.P.D., that of pepsin was 10·0, and that of gastricsin was 1·88. About 85% of applied activity was routinely recovered.

3.2 Chromatography of pepsin and gastricsin on DEAE-Sephadex A50

As described in the Introduction to this work, a number of workers have identified more than two human pepsins. Also, it has been reported that human gastricsin does not hydrolyse A.P.D. (Chiang et al 1966),/...

al, (1966), contrary to the results shown in the preceding Section. Thus, it appeared possible that the chromatography on Amberlite IRC 50 was not providing sufficiently sensitive separation of pepsins. Because of this, it was decided to try to fractionate further the pepsin and gastricsin obtained. Initially, DEAE-cellulose was used. It was found that, using 0·1 M citrate buffers of pH 3·2 and pH 3·6 , the enzymes did not bind to the resin, although chromatography at pH 3·2 was used to purify pig pepsins (Lee & Ryle 1967). Although the enzymes would bind at pH 4·0, using 0·1 M acetate buffer, results were not very satisfactory, poor resolutions being obtained. Various changes in the gradients of NaCl used made no difference to this. Considerable improvement in resolution was found when DEAE-Sephadex A50 was used; the results of these experiments are detailed below.

Amberlite IRC 50

Freeze-dried pepsin obtained from two separate chromatographies of human gastric juice were pooled, and 20·5 mg of this was dissolved in 0·1 M acetate buffer, pH 4·0, to a concentration of approximately 10% (w/v) and centrifuged and prepared as described in Sect. 2.6, where full details of the chromatographic procedure are given. The supernatant was applied at 4° C to a 2·5 x 17 cm column of DEAE-Sephadex A50, prepared and equilibrated as described earlier (Sect 2.6) and the chromatogram was developed at 4° C. All buffers were prepared, and their pHs were measured, at room temperature and they were then used at 4° C. After completion of the chromatographies, the resin was regenerated <u>in situ</u> by washing with NaCl-free buffer, until the effluent was free from detectable NaCl and the resin had swollen to its original height.

After/.....

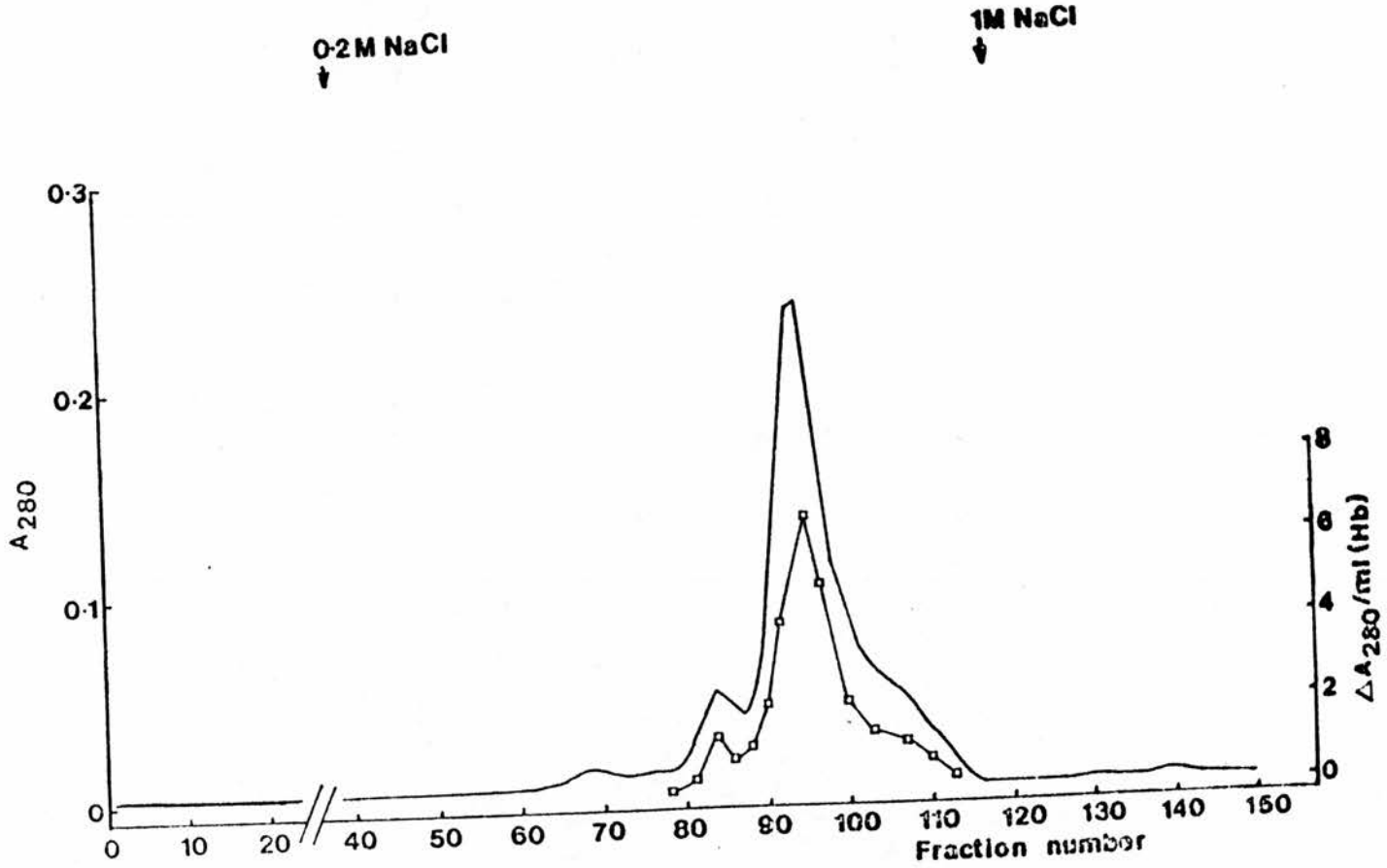

Fig. 2. Chromatography of pepsin on DEAE-Sephadex A50, using 0·1 M acetate buffer pH 4·0. Arrows indicate introduction of exponential NaCl gradients; volume of mixing vessel, 100 ml. Fractions were 5 ml. Column 2·5 cm x 17 cm, 20 ml/hr. —, protein; □, haemoglobin (Hb) assay. Gradients were produced by passing buffer containing Na Cl from the open reservoir into the closed mixing vessel.

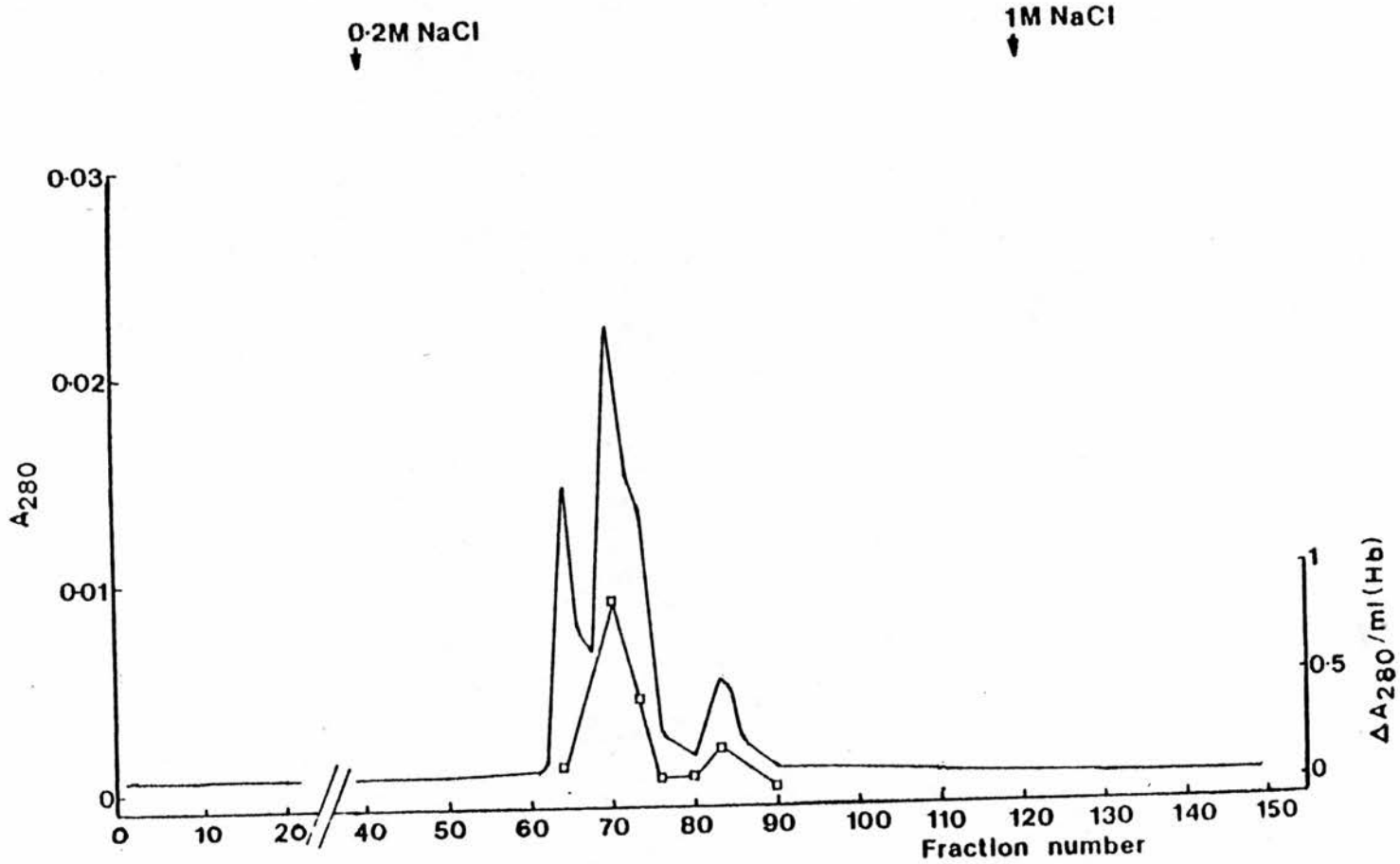

Fig. 3 Chromatography of gastricsin on DEAE-Sephadex A50; conditions as in Fig. 2. Arrows indicate introduction of NaCl gradients. —, protein; □, haemoglobin (Hb) assay.

After permeation of the sample into the resin bed, 0·1 M acetate buffer, pH 4·0, was applied until the absorbance of the eluate at 280 nm was zero. Fractions of 5 ml were collected. Buffer was applied by positive pressure at a flow-rate of 20 ml/hr.

The chromatogram was further developed using exponential gradients of NaCl. Details concerning the production of these gradients are given in Sect. 2.6.

The elution profile is shown in Figure 2. It appears that the pepsin peak obtained from the Amberlite column was not homogeneous. There appear to be three quite distinct peaks, plus a shoulder on the main peak, eluted by the gradient up to 0·2 M NaCl. There also appears to be at least one peak eluted in the gradient up to 1 M NaCl, although this is not easy to detect, because of the low absorbancies involved. The enzymic activity of the major peaks was measured using haemoglobin as substrate, as described in Sect. 2.2. The specific activities of these major peaks, in order of elution, were 0,23·6 and 27·8; that of the shoulder 25·0.

The gastricsin obtained from the Amberlite column was treated in a similar fashion, a total of 9·3 mg of freeze-dried material being used. The elution profile is shown in Figure 3. Three peaks were eluted by the gradient up to 0·2 M NaCl. There did not appear to be any protein in fractions eluted by the gradient up to 1 M NaCl. It may be seen that the first peak eluted had virtually no haemoglobin-digesting activity. The second peak had a specific activity of 38·3, while that of the third peak was 34·6. The elution position of the third peak from the chromatogram of gastricsin appears to coincide with that of the first peak in the chromatogram of pepsin. Given that the pepsin and gastricsin peaks on Amberlite were well-resolved, this could reflect micro-

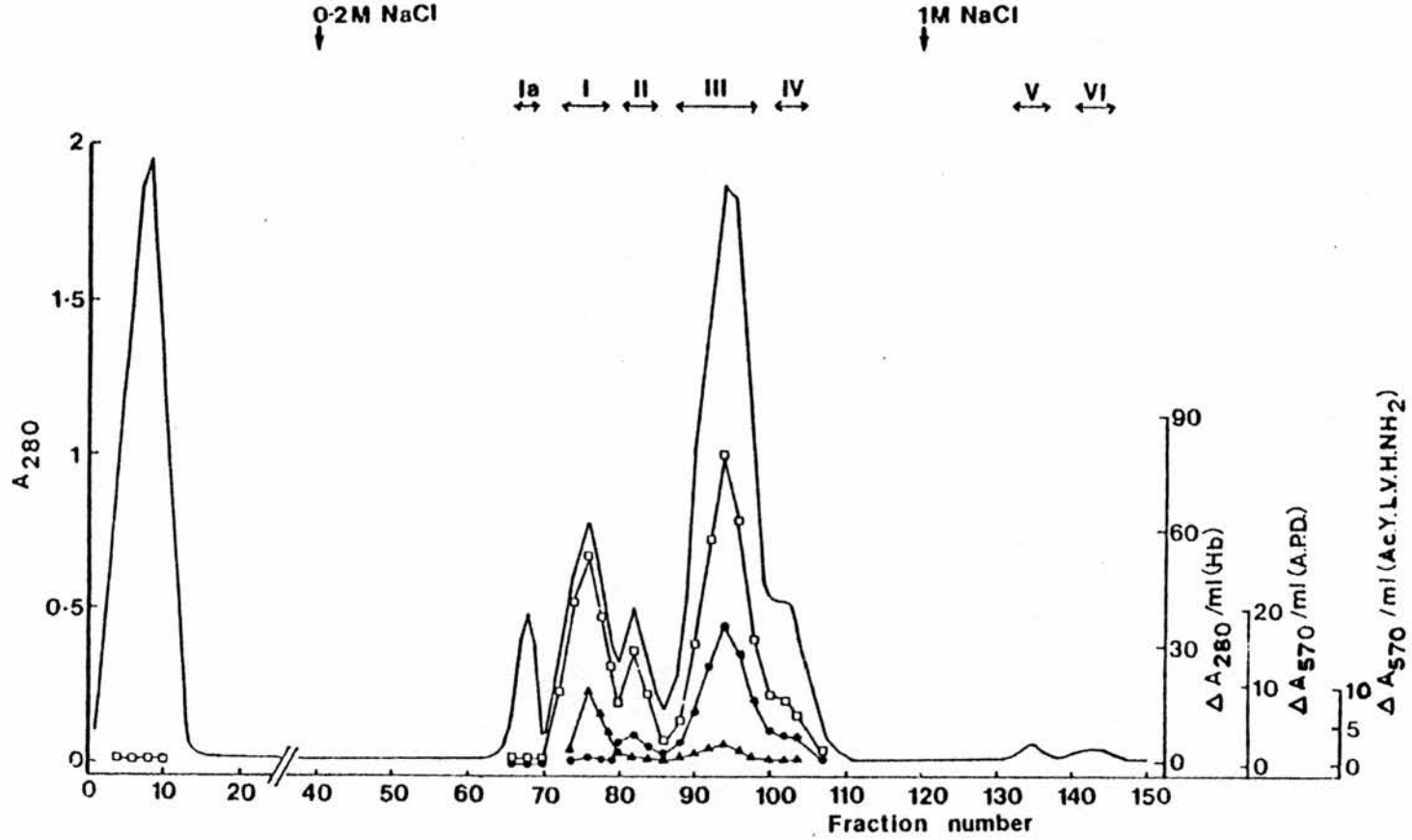

Fig. 4 DEAE-Sephadex A50 chromatography of human gastric juice, conditions as in Fig. 2. Arrows indicate introduction of NaCl gradients. Fractions were pooled as shown. — , protein; □, haemoglobin (Hb) assay; ●, A.P.D. assay; ▲, Ac.Y.L.V.H.NH_2 assay.

heterogeneity within each peak. It would have been preferable, in order to test this, to pool the peaks and re-chromatograph them on Amberlite. The occurrence of one peak or two would have shown identity or distinction.

3.3 Chromatography of human gastric juice on DEAE-Sephadex A50

Fractionation of pepsin and gastricsin, obtained by chromatography on Amberlite IRC 50, on DEAE-Sephadex A50 clearly showed that neither was homogeneous. Both samples contained appreciable amounts of other proteolytically-active proteins. Clearly it is necessary to obtain as good a resolution of enzymes as possible. Therefore, in order to study further the properties of these proteins, human gastric juice was fractionated on DEAE-Sephadex A50. It may have been useful to use preliminary fractionation of gastric juice on Amberlite, followed by fractionation of pepsin and gastricsin on DEAE-Sephadex A50. This would permit detailed study of any micro-heterogeneity within the Amberlite peaks.

Gastric juice was collected and prepared for chromatography as detailed in Sect 2.1, except that the freeze-dried material was dissolved in 0·1 M acetate buffer, pH 4·0. Samples were obtained from patients and volunteers. Chromatograms were developed as described in Sect. 2.6. As explained in that Section, application of freeze-dried gastric juice from more than five subjects to a 2·5 cm x 17 cm column tended to overload the column.

Figure 4 shows a typical elution profile for gastric juices pooled from several subjects. A large peak of enzymically-inactive material was removed in the NaCl-free wash. The properties of this material were not studied. Most of the active material was eluted in the gradient up to 0·2 M NaCl, well separated from the inactive material. In the diagram the individual points on the line representing protein have been omitted for clarity; in practice the absorbance at 280 nm of each fraction was determined. Figure 4 represents/....

Table 2 Specific activities of peaks obtained by chromatography of human gastric juice on DEAE-Sephadex A50, as shown in Figure 4.

Peak	Haemoglobin	A.P.D.	Ac.Y.L.V.H.NH_2
Ia	0	0	0
I	82	1·6	10·7
II	52	7·6	see text
III	39·5	9·2	0·85
IV	30·8	6·9	0·36
V	33	2·5	0
VI	35	2·7	0·17

represents a chromatogram of pooled gastric juices from four subjects, all of whom were suffering from duodenal ulcers. The peaks of interest have been labelled in order of their elution from the column.

A total of seven such chromatograms was performed, using gastric juice from more than one subject. Table 2 shows the specific activities of the various peaks shown in Figure 4 using haemoglobin, A.P.D. and Ac.Y.L.V.H.NH_2. The specific activities are expressed in the units defined in Sect. 2.

3.3.1 Isolation of peak Ia

Peak I a was found in all samples. Most such peaks were found to be inactive against all substrates used, although the assay with Ac.Y.L.V.H.NH_2 is not shown in Figure 4 for clarity. Occasionally some fractions in peak Ia showed very low specific activities against haemoglobin of about 3-6, perhaps due to contamination from another peak or peaks although in most cases peak Ia was inactive. The material in peak Ia did not clot milk, the assay being performed as detailed in Sect. 2.5, using a protein solution of 0·1 mg/ml. The nature of this material was thus unclear; the ratio of A_{280}: A_{260} was found to be 0·9. From a standard table prepared using yeast enolase and yeast nucleic acid, this value indicates that the sample is 95% protein (Data for Biochemical Research, 1969). Although this technique is liable to error because other proteins and nucleic acids have different absorbancies, it would seem reasonable to conclude that peak Ia is a protein.

3.3.2 Isolation of peak I

Peak I has relatively low peptidase activity against A.P.D. Although/...

Although this activity is low, its profile follows that of the protein peak, so it may represent genuine activity rather than contamination from other peaks. This enzyme also has the highest specific activity in the haemoglobin-digesting assay. These properties appear to be characterisitic of human gastricsin. Table 2 also shows that peak I has the highest specific activity against Ac.Y.L.V.H.NH_2. Thus, peak I appears to be equivalent to human gastricsin and closely related to pig pepsin C. Peak I was found in all gastric juices, from healthy volunteers and from patients with ulcers. In all cases, peak I was the second most abundant protease in gastric juice.

3.3.3 Isolation of peak II

The minor peak II was also present in all chromatograms. Table 2 shows that the enzyme readily hydrolyses haemoglobin and A.P.D. However, although fractions under peak II were found to hydrolyse Ac.Y.L.V.H.NH_2 there appears to be a fall of this activity across the protein peak. Thus, this activity might not be a property of peak II, but could represent the presence of contaminating peak I. For convenience, and in order to conserve Ac.Y.L.V.H.NH_2 which had to be synthesized in the laboratory, peptidase activity was not determined for every fraction in all chromatograms. The activity of all fractions in peak II against Ac.Y.L.V.H.NH_2 was determined in only two chromatograms of gastric juice but the fall of activity was seen in both cases. This effect can be more clearly seen in subsequent re-chromatography of this protein. (Sect. 3.5.3).

3.3.4 Isolation of peak III

Peak III was found to be the most abundant pepsin in all gastric juices. On this basis, it is probably equivalent to pepsin 3 reported/....

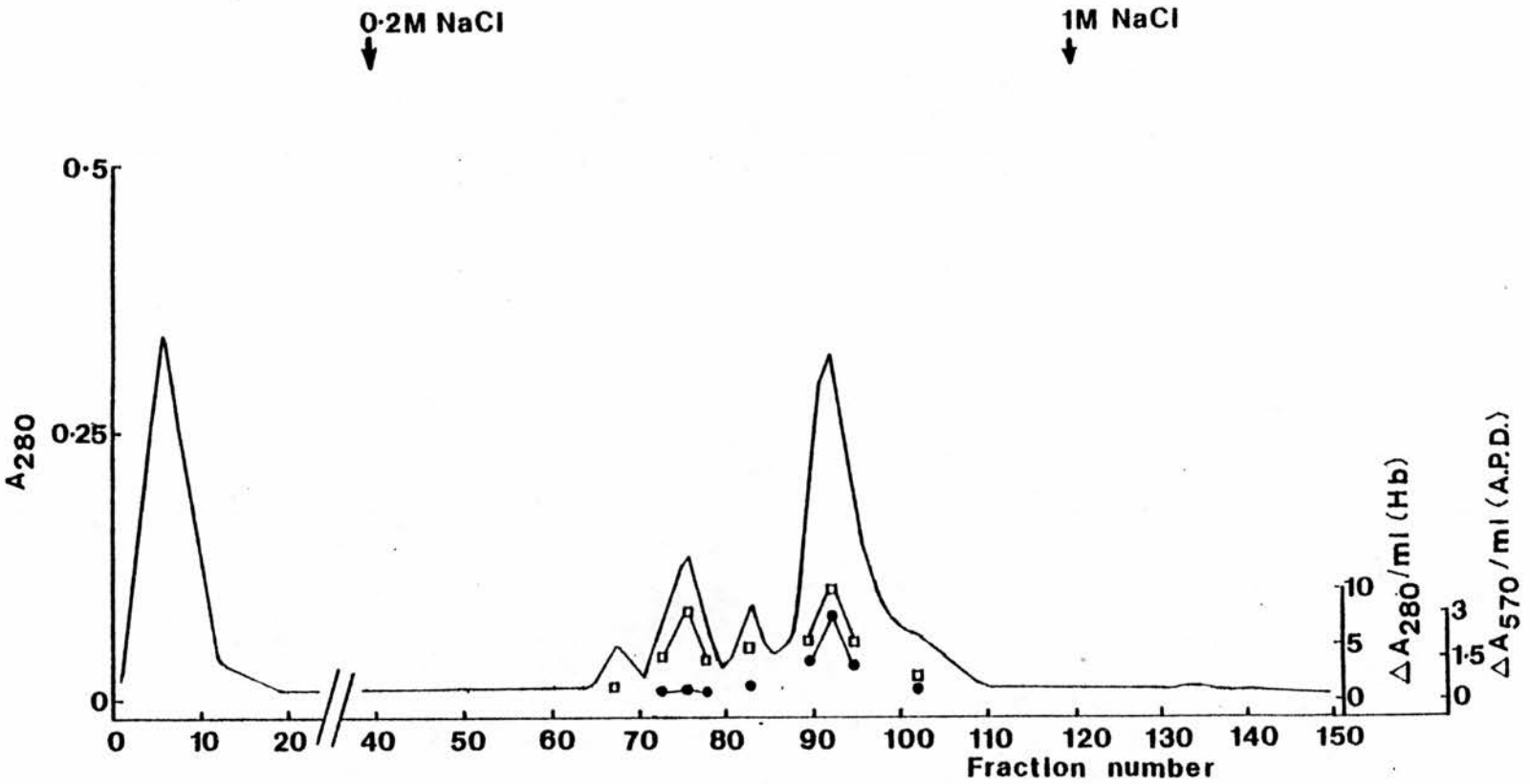

Fig. 5 DEAE-Sephadex A50 chromatography of gastric juice from a healthy volunteer, conditions as in Fig. 2. Arrows indicate introduction of NaCl gradients. Symbols as in Fig. 4.

reported by Taylor and his colleagues (e.g. Roberts & Taylor, 1978a). Further consideration of this point is given below (Sect. 3.5.4.). The separation of peak III from peak I reported here represents a considerable improvement in resolution compared to that shown by Roberts & Taylor (1978a). Peak III appears to have activity against all substrates used.

3.3.5 Isolation of peak IV

Peak IV occurred as a well-defined shoulder in all pooled gastric juices examined. Etherington and Taylor (1969) reported the detection of an electrophoretic zone, pepsin 3a which has a slightly greater mobility than their pepsin 3. Roberts and Taylor (1978a) showed that pepsin 3 a is eluted from DEAE-cellulose just after pepsin 3. It may be that peak IV is equivalent to pepsin 3a, although this is only a tentative suggestion at this stage.

3.3.6 Isolation of peaks V and VI

Peaks V and VI were found in all samples of gastric juice pooled from more than one subject. Table2 shows some specific activities for these peaks. It is not clear whether these enzymes, especially peak V, have peptidase activity against Ac.Y.L.V.H.NH_2. Some fractions showed such activity, albeit very low, whilst others did not. The fact that the absorbancies at 570 nm being determined for these fractions were so low may throw some doubt on these results. Even in those samples which showed no such activity this may be due to the extremely low concentrations of protein.

3.3.7 Fractionation of gastric juice from healthy volunteers

Figure 5 shows the elution pattern for gastric juice from a volunteer/...

Table 3 Some specific activities for peaks obtained by chromatography on DEAE-Sephadex A50 of gastric juice from a healthy volunteer. See Figure 5.

Peak	Haemoglobin	A.P.D.
Ia	0	N.D.
I	65·5	0·47
II	42·0	5·6
III	30·3	9·3
IV	34·0	4·5
V	36·0	N.D.
VI	34·5	N.D.

N.D. = not determined.

volunteer who did not have any known gastric or duodenal disease. The similarity of this profile with that of Figure 4 is clear, although peaks V and VI are more prominent in Figure 4 , the latter representing the chromatogram of a much larger volume of gastric juice. It may be noted that the various peaks were eluted at approximately the same positions in both experiments. Selected fractions were assayed for digestion of haemoglobin and hydrolysis of A.P.D. The results are shown in Figure 5; these data are also given in Table 3. Those specific activities which were determined are broadly similar to those in Table 2. Concentrations of peaks V and VI in this chromatogram were thought to be too low for reliable peptidase assay.

Five gastric juice samples taken from individuals without duodenal ulcers were fractionated. Of these, two were volunteers and three were from suspected ulcer sufferers who proved on endoscopy to be free from gastric or duodenal disease. In all cases peaks Ia - IV were clearly present; peak Ia was found to be enzymically inactive.

Peaks V and VI were not always simultaneously present. During fractionation of gastric juice from a single subject it was frequently difficult to determine whether or not a genuine peak was present. Also, in some cases what appeared to be a single broad peak covering the range of elution of peaks V and VI was observed, making discrimination of peaks impossible.

3.3.8 Fractionation of gastric juice from individuals with duodenal ulcer

Gastric/...

Gastric juice samples from six patients suffering duodenal ulcers were fractionated separately. The elution profiles were very similar to that shown in Figure 5. Peaks Ia - III were found in all six patients, peak IV being absent from only one subject, a 51 year old female.

It must be stressed that, in the fractionation of gastric juice from individuals with or without ulcer, the detection of peaks V and VI was generally quite difficult, as is explained above (Sect. 3.3.7). Thus it was felt that chromatography was probably not sensitive enough to provide much evidence regarding any differences in secretion patterns between healthy subjects and those with ulceration.

It may also be noted that, in the relatively small number of individual samples examined, there were no obvious differences in secretion patterns due to either sex or age differences.

3.3.9 Effect of pentagastrin

As mentioned in Sect. 2.1, pentagastrin was routinely used to stimulate secretion of gastric juice. In order to determine whether pentagastrin had any qualitative effect on secretion the following experiment was performed using gastric juice from two subjects, one having duodenal ulcer, the other being a healthy volunteer. After overnight fasting, the overnight secretion plus the basal secretion over one hour were aspirated and pooled. Pentagastrin was administered by nursing staff, and the stimulated secretion was aspirated for a further hour, this being kept separate from the basal plus overnight secretion. The samples were then prepared and chromatographed separately on DEAE-Sephadex/...

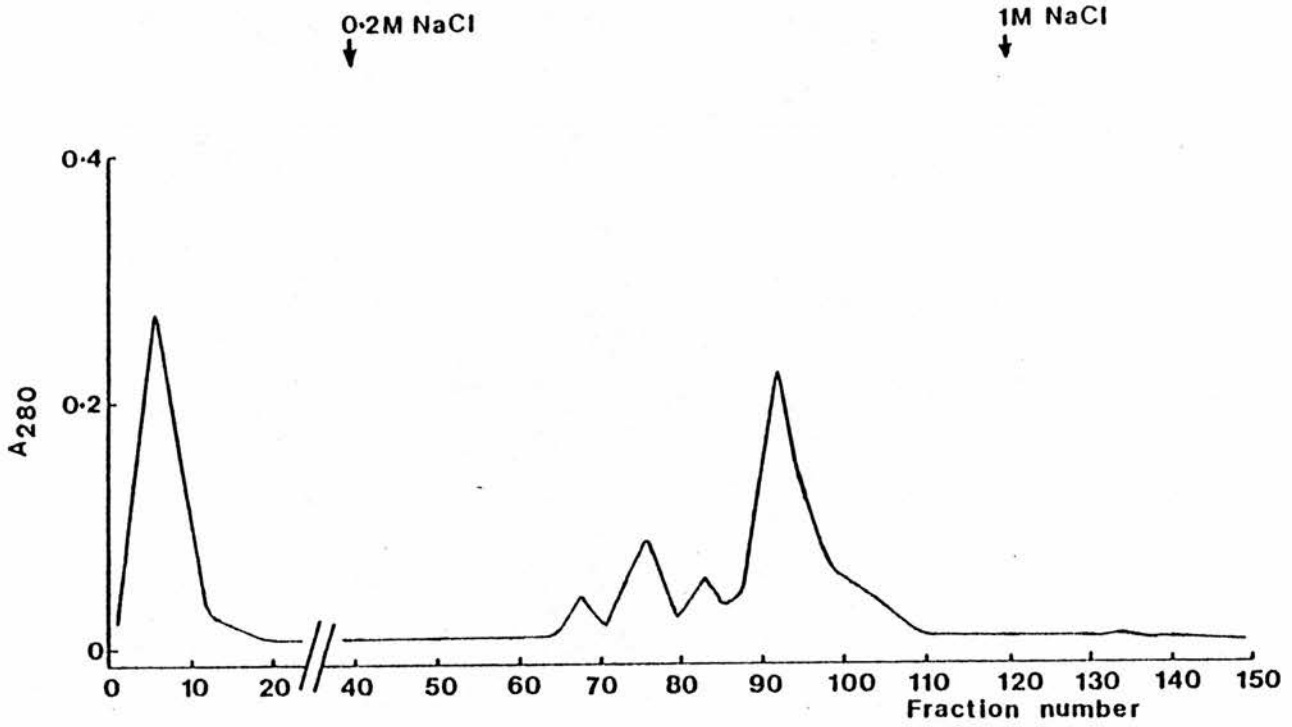

Fig. 6 DEAE-Sephadex A50 chromatography of gastric juice, conditions as in Fig. 2. Arrows indicate introduction of NaCl gradients. — , protein.

on DEAE-Sephadex A50, as already described. For both subjects basal and pentagastrin-stimulated gastric juices yielded elution profiles qualitatively the same as that in Figure 5. Specific activities of the individual peaks were not determined. It thus appears that pentagastrin does not affect the qualitative secretion of pepsins, and all further work was performed on pooled basal plus stimulated secretions.

3.3.10 Influence of Autolysis

A typical chromatography of the type described took three days to complete. During this time the pepsins were at pH 4·0 - 4·7 (see below), in which range autolytic breakdown occurs. To determine if any of the peaks might arise from autolysis of other peaks, one sample of gastric juice pooled from two subjects was prepared as described in Sect. 2.1. Freeze-dried material was dissolved in 0·1 M acetate buffer, pH 4·0, centrifuged and divided into two equal portions. One was chromatographed immediately; the elution profile is shown in Figure 6. The other portion was stored at 4° C for three days and then chromatographed; its elution profile was qualitatively the same as that in Figure 6. There were no obvious changes in the relative sizes of the peaks thus indicating that, under the conditions of this experiment, the occurrence of any of the peaks could not be explained by autolysis. Obviously this does not eliminate the possibility of such autolysis in the stomach or during aspiration when the gastric juice is at a much lower pH, under which conditions the pepsins would be more proteolytically active than at pH 4·0 and 4° C.

In a further experiment, 15 mg of a sample of peak III purified/...

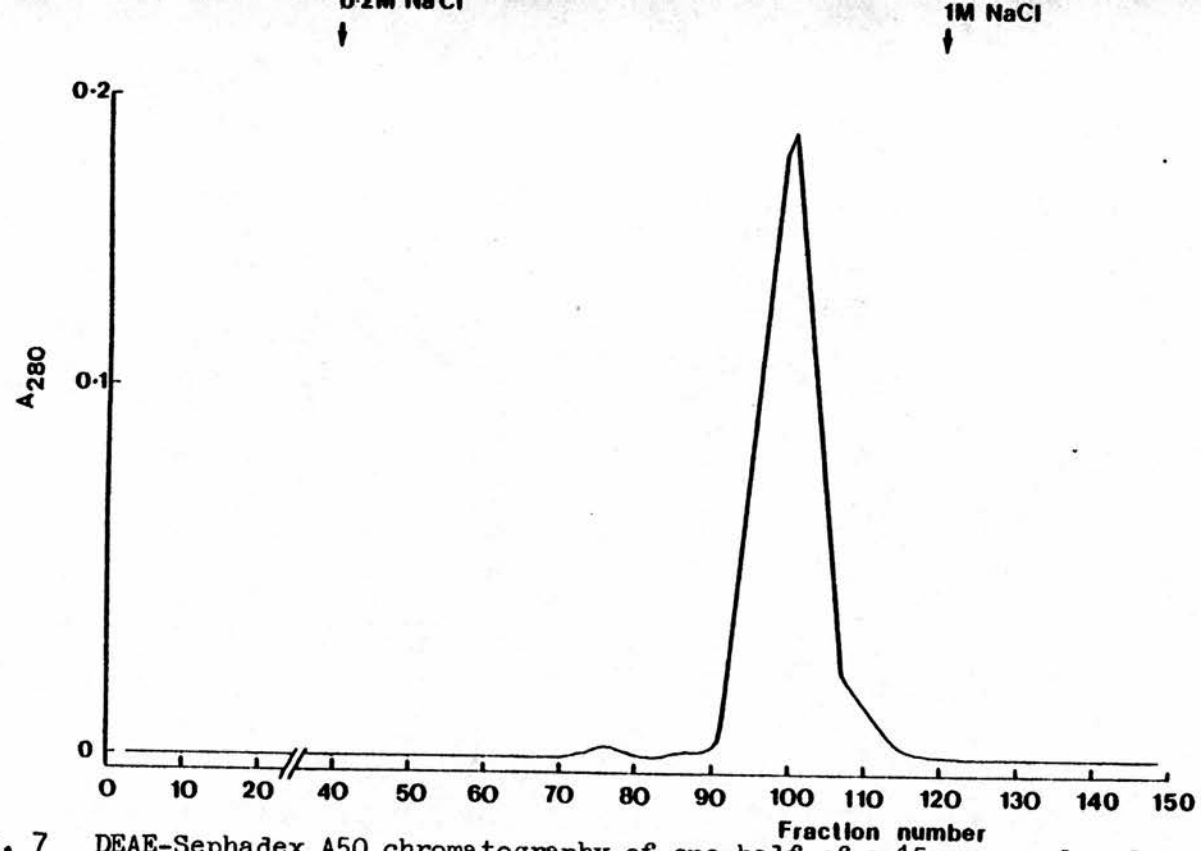

Fig. 7 DEAE-Sephadex A50 chromatography of one-half of a 15 mg sample of peak III. Conditions as in Fig. 2. Arrows indicate introduction of NaCl gradients. ——, protein.

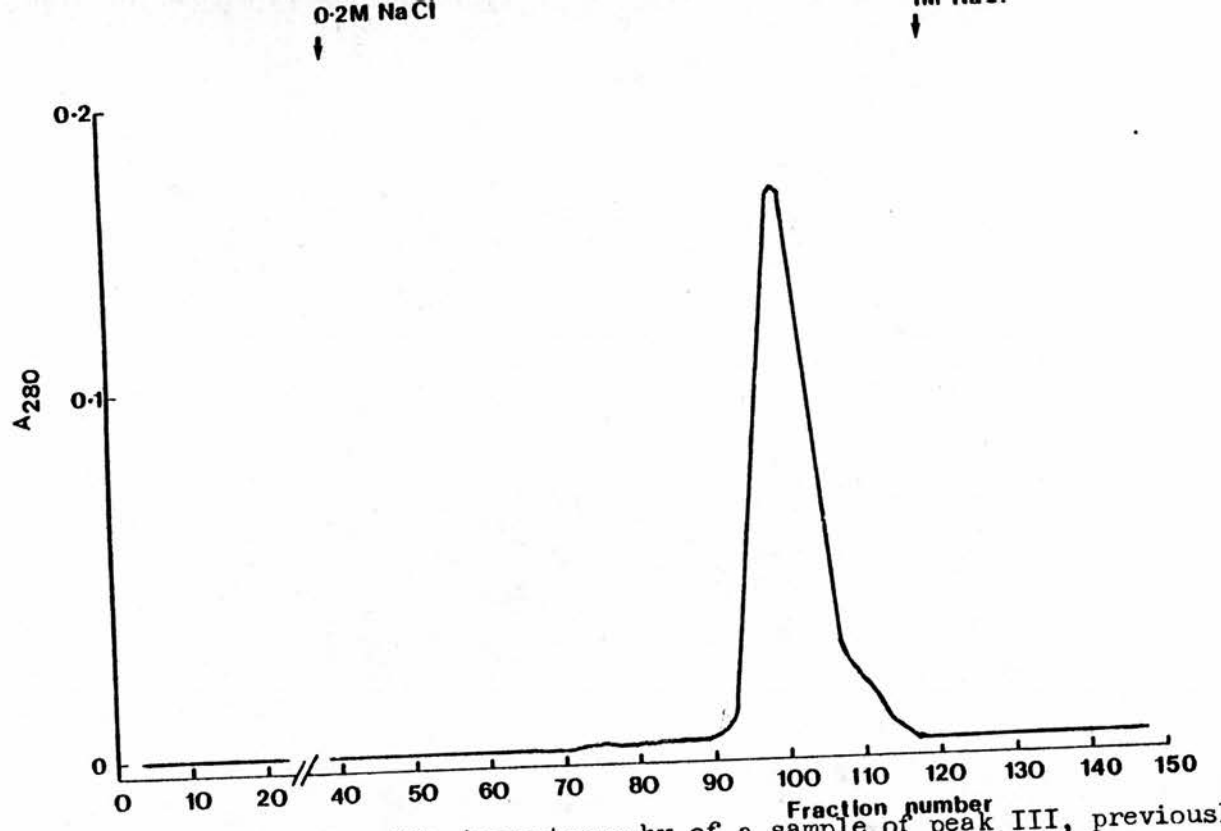

Fig. 8 DEAE-Sephadex A50 chromatography of a sample of peak III, previously incubated at pH 1·2, room temperature, four hours. Conditions as in Fig. 2. Arrows indicate introduction of NaCl Gradients. —, protein.

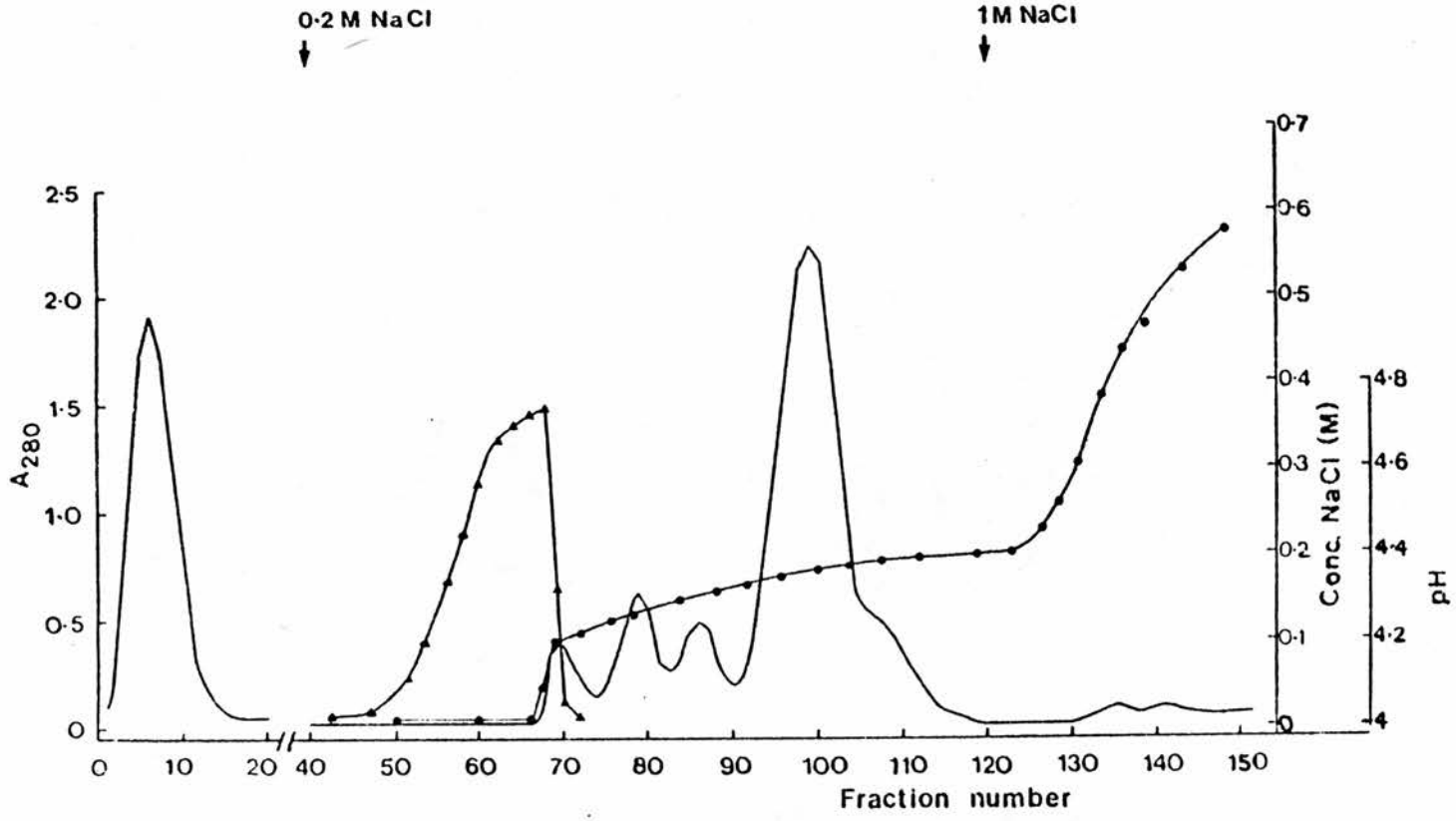

Fig. 9 DEAE-Sephadex A 50 chromatography of human gastric juice, conditions as in Fig. 2. The elution profile shown in relation to NaCl gradient and pH of fractions, ▲ pH; ● NaCl concentration.

purified by one chromatography on DEAE-Sephadex A50 was dissolved in 1·5 ml of distilled water and centrifuged in an MSE 18 at 20,000 g for 15 minutes. The solution was divided into two portions, to one of which was added sufficient 2 M acetate buffer, pH 4·0, to make the final solution 0·1 M acetate. This was chromatographed on DEAE-Sephadex A50 as already described. The other protein was titrated to pH 1·2 with HCl and allowed to stand at room temperature for four hours, after which it was dialysed against several changes of 0·1 M acetate buffer pH 4·0 and chromatographed on DEAE-Sephadex A50. Figure 7 shows the elution profile of the sample which was chromatographed without titration to pH 1·2; Figure 8 shows that of the sample incubated at pH 1·2. Comparison of the diagrams shows no significant change in size of any of the peaks. Thus, during incubation at pH 1·2 under the conditions used, autolysis of the major peak does not appear to give rise to formation of any of the other peaks.

Even if the existence of some peaks can be explained by autolysis under physiological conditions, they could still have a role in the aetiology of ulceration. However, it does appear that none of the peaks is an artifact of the chromatographic procedure. Determination of activities across the peaks may have helped show whether autolysis occurred without forming extra peaks.

3.3.11 Gradient of NaCl and change in pH during chromatography

Figure 9 shows the gradient of NaCl and the pH of the fractions for a typical chromatogram. No chloride ions would be expected in fractions representing the 'dead volume' of the column, and it can be seen that there is virtually no NaCl in the first 25 fractions following application of 0·2 M NaCl; this represents

both/....

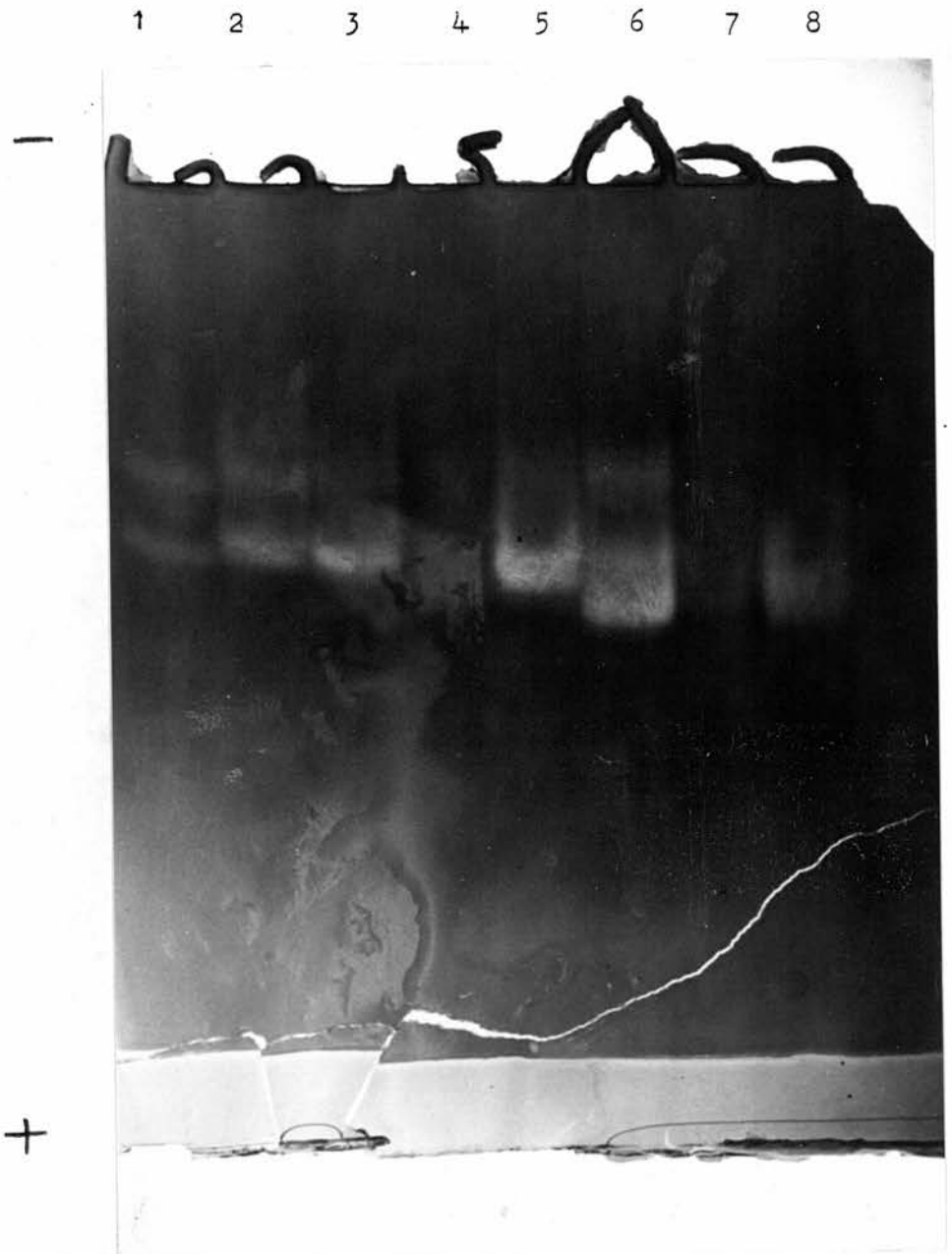

Plate 1. Electrophoresis of pepsins at pH 4·0 in 7·5 % acrylamide gel. Human pepsins were obtained by DEAE-Sephadex A50 chromatography of gastric juice. Electrophoresis was conducted at 150 v for $4\frac{1}{2}$ hrs. The gel was stained for activi

slot	1	2	3	4	5	6	7	8
sample	I	II	III	-	IV	V	VI	pig pepsin

both the 'dead volume' and the displacement of acetate ions from the gel beads by chloride ions. The rise in pH is presumably due to the appearance of increased levels of acetate ions in the effluent. This pH rise is not high enough to denature pepsins, a process which generally occurs at pH values above pH 6.

3.4 Electrophoresis in polyacrylamide gels

Following preparation of pepsins as described above, it was necessary to determine whether or not each sample was homogeneous before further studies could be performed. In preliminary experiments, electrophoresis was performed in gels of 6% acrylamide, 0·01 M acetate buffer pH 5·6, cast in 0·5 x 7·5 cm glass tubes. Poor separation resulted, and in some cases the bromophenol blue marker front did not move at the same rate in different gels, making the gels difficult to compare. This technique was therefore abandoned, and slab gels were used as described in Sect. 2.8.1. Separation was thus improved, an important consideration when one is dealing with a number ofproteins with similar properties. Also, a number of samples can be applied to the same gel, helping to ensure uniformity of conditions.

Plate 1 shows a polyacrylamide gel run in a slab using the system in which a stacking gel is omitted. Solutions of freeze-dried samples obtained from chromatography of gastric juice were used, solutions being 0·1 mg/ml. These were centrifuged and prepared for electrophoresis by the addition of bromophenol blue and sucrose as described in Sect 2.8.1; 10 μl of each solution was applied to the gel. Electrophoresis was performed at constant voltage/...

Table 4 Anodal mobilities of human pepsins separated by polyacrylamide gel electrophoresis, compared to pig pepsin.

Pepsin	Relative mobility
pig	1
I	0·69
II	0·87
III	0·89
IV	0·94
V	1
VI	0·97

voltage, 150 v for $4\frac{1}{2}$ hours. Under these conditions the gels did not overheat, so a cooling system was not required. A sample of pig pepsin (10 μl of 0·1 mg/ml solution) was also applied as a marker. It may be seen from Plate 1 that there are detectable levels of slow-running material in samples II-V. In track 1, there are two bands of equal intensity. It may be seen from other gels (e.g. Plate 4) that peak I is the slowest band, and it is this band which has the relative mobility of 0·69 that is given in Table 4. It is clear that pig pepsin has a greater mobility than peak III, the major human pepsin which is frequently regarded as the nearest human equivalent to pig pepsin A. In this gel, the band of peak VI is comparatively faint. As is explained below, purification of peak VI was extremely difficult. It could be that in the sample used here a high proportion was inactive material, possibly denatured by freeze-drying. Alternatively, peak VI may have lower haemoglobin-digesting ability than the other pepsins, although data in Table 2 suggest this is probably not the explanation.

The mobilities of the pepsins correspond well with their order of elution from DEAE-Sephadex A50. Thus, peak I is clearly the least electronegative and is the first eluted from the ion-exchange column. Peaks II-IV are closely clustered in the electrophoresis, and this is also found in the chromatography, especially for peaks III and IV. It appears from Plate 1 that peak V has a slightly greater mobility than peak VI which is somewhat surprising in view of their order of elution. Electrophoretically and chromatographically peaks V and VI are distinct from the other peaks but similar to each other.

Table 4 shows the relative mobilities of the pepsins compared to that of the pig pepsin marker.

3.5 Rechromatography of proteins on DEAE-Sephadex A50

Electrophoresis of the individual enzymes showed they were not homogeneous and that therefore a single chromatographic step was not sufficient. Because good resolution had previously been obtained using DEAE-Sephadex A50, it was thought that this ion-exchanger would be suitable for further purification.

Dialysed, freeze-dried peaks obtained after fractionation of human gastric juice on DEAE-Sephadex A50, as described in Sect. 3.3, were dissolved in 0·1 M acetate buffer pH 4·0, centrifuged and re-chromatographed by the same procedure.

3.5.1 Rechromatography of peak Ia

A porcine pepsin which has very little activity against haemoglobin but has high activity against A.P.D. has been described (Ryle & Porter 1959); it was named pepsin B. In the chromatography of crude pig pepsin on DEAE-cellulose at pH 4·0, this was the first active peak to emerge. Thus, although peak Ia is apparently inactive against A.P.D. and Ac.Y.L.V.H.NH_2 and has little, if any, activity against haemoglobin, it is just possible that this material is actually a pepsin of somewhat different specificity from that of the other pepsins. Obviously peak Ia has one characteristic in common with the human pepsins, as shown by its acidic nature. Therefore it was thought that further purification and amino acid analysis might give some useful indications regarding the nature of peak Ia, so the material was subjected to a purification procedure similar to that used for the other chromatographic peaks described here.

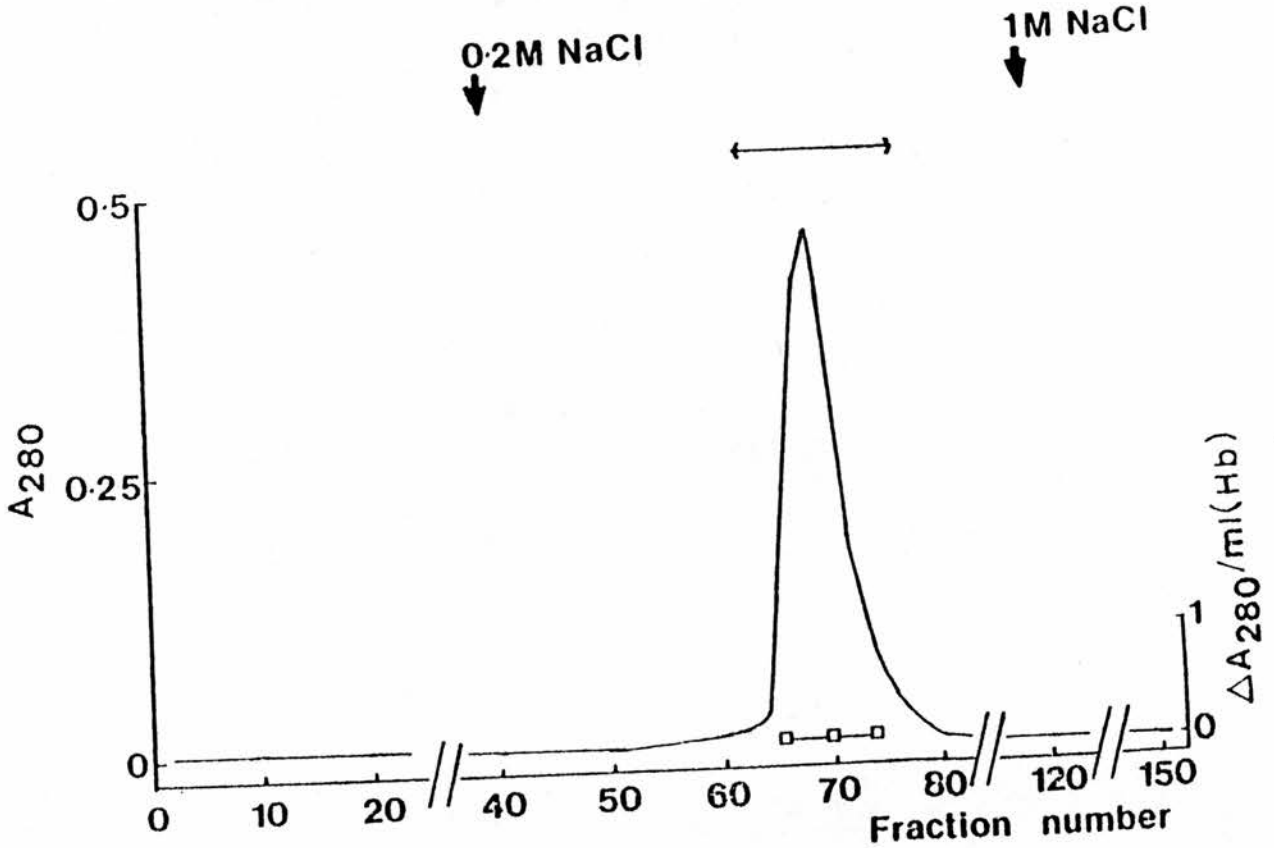

Fig 10 Rechromatography of Ia on DEAE Sephadex A50 buffer pH 4·0. Arrows indicate introduction of NaCl gradient. Fractions (5 ml) were pooled as shown.

—, protein

□, assay with haemoglobin

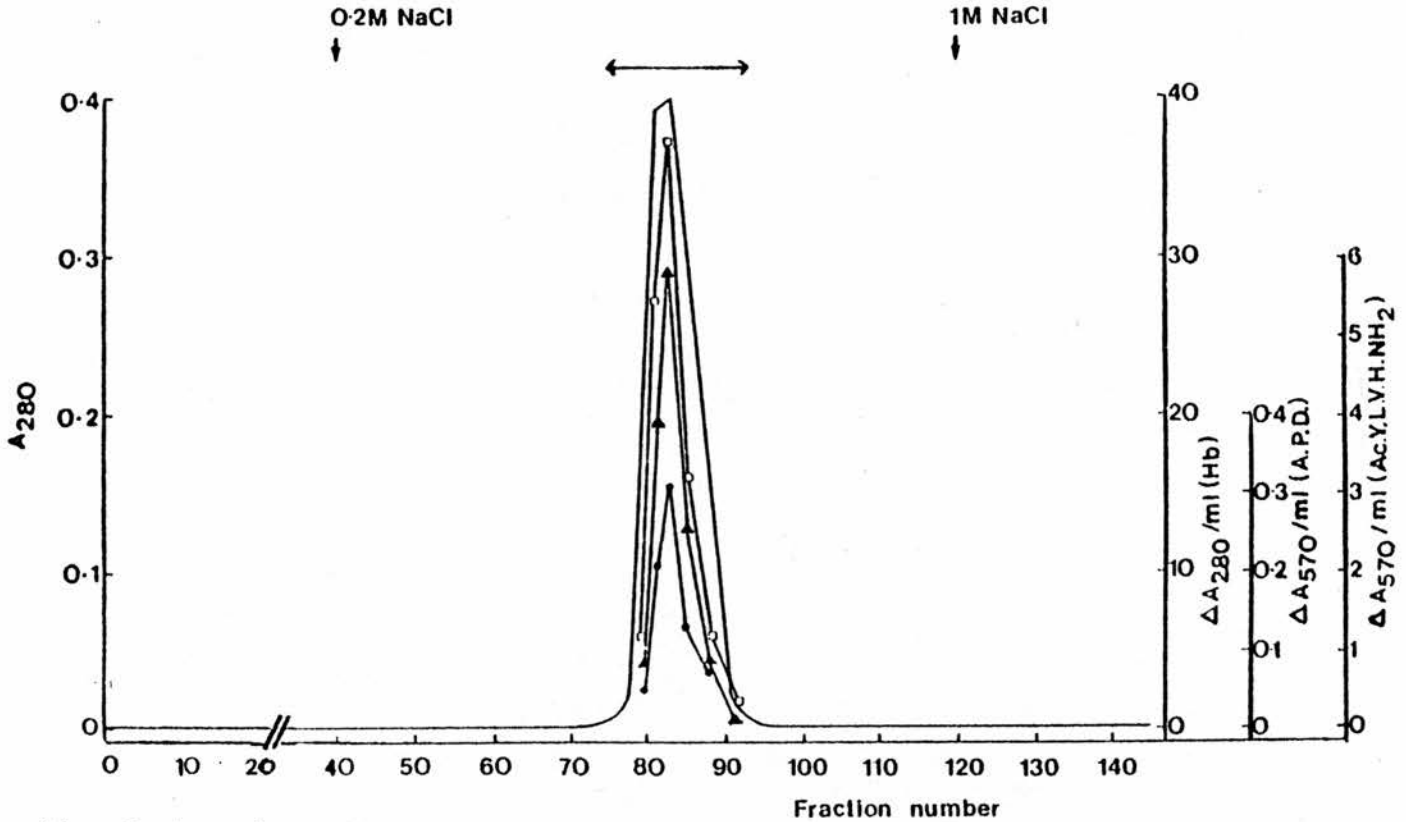

Fig. 11 Rechromatography of peak I on DEAE-Sephadex A50, conditions as in Fig. 2. Arrows indicate introduction of NaCl gradients. Fractions were pooled as shown. — protein (A_{280}); □, haemoglobin assay; ▲, Ac. Y.L.V.H.NH_2 assay; ●, A.P.D. assay.

A sample of 21 mg of peak Ia was used, this being pooled from two separate chromatographies of gastric juice. Figure 10 shows the elution profile obtained for this experiment. Fractions which were pooled, dialysed and freeze-dried are shown. As may be seen, there is no obvious presence of any other peak, although it is noticeable that the peak is not symmetrical. This may indicate the presence of some contaminating material. No enzymic activity was detected. All three substrates (haemoglobin, A.P.D. and Ac.Y.L.V.H.NH_2) were used, although for clarity only the data for haemoglobin are shown in Figure 10. The peak was eluted at approximately the same position as in chromatograms of gastric juice.

3.5.2 Rechromatography of peak I

Peak I(13·5 mg of freeze-dried material) taken from the chromatogram shown in Figure 4 was used. It should be noted that the peak I shown here is not the same preparation as that in track 1 of Plate 1, otherwise one would expect more than one peak in Figure 11, which shows the elution profile, along with the results of assays with haemoglobin, A.P.D., and Ac.Y.L.V.H.NH_2. Fractions which were pooled, dialysed and freeze-dried are shown. Specific activities are given in Table 5. There is no obvious presence of any other peak, but it is clear that peak I shows activity against both peptide substrates as well as haemoglobin. The specific activities of peaks II-IV against A.P.D. are in the approximate range 3-12, while that of peak I is about 0·9. Thus, if peak I actually has no such activity one must postulate that about 10% of the material appearing in peak I is one or more of the other, A.P.D. - hydrolysing peaks. Given the clear separation of peak I from the other peaks shown in Figures 4 and 5, it would/...

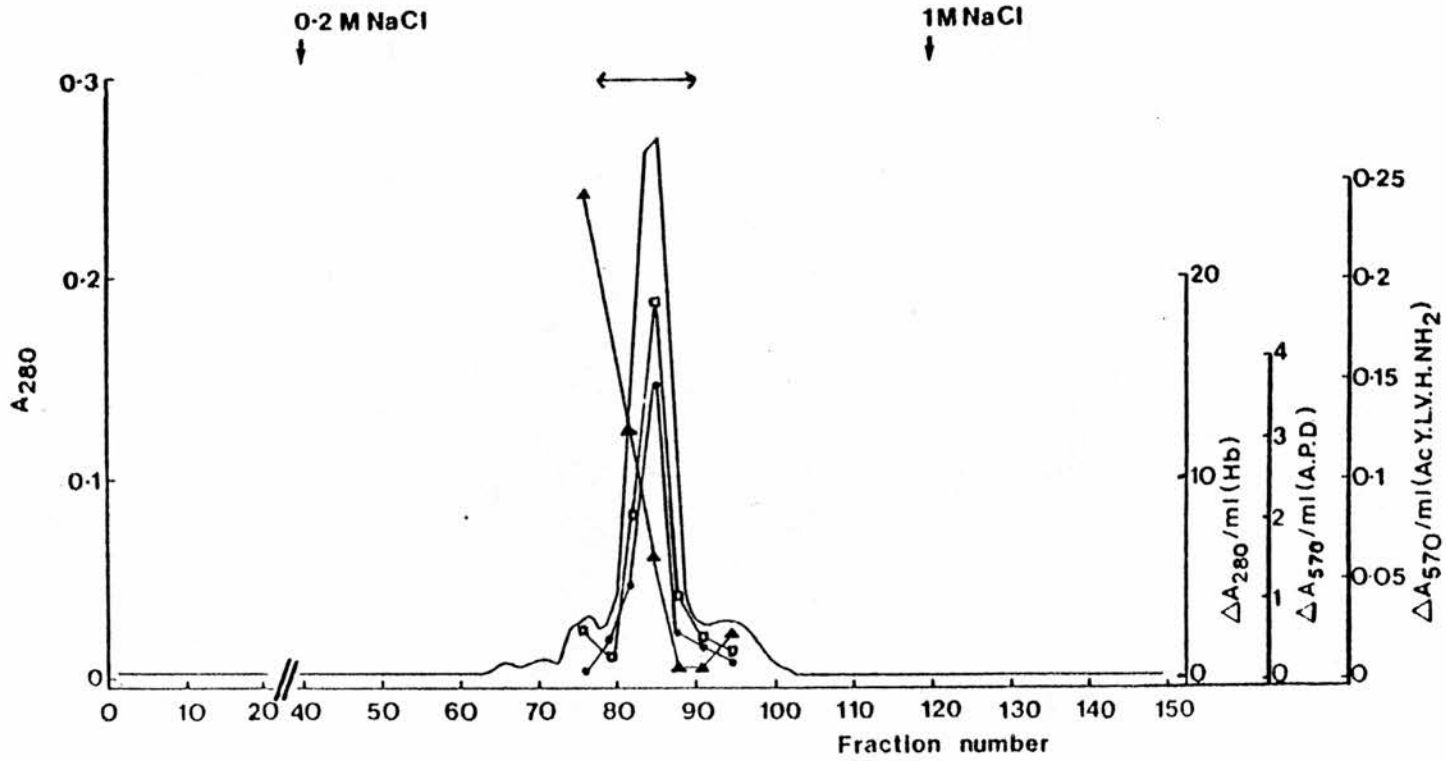

Fig. 12 Rechromatography of peak II on DEAE-Sephadex A50, conditions as in Fig 2. Arrows indicate introduction of NaCl gradients. Fractions were pooled as shown. Symbols as in Fig.11.

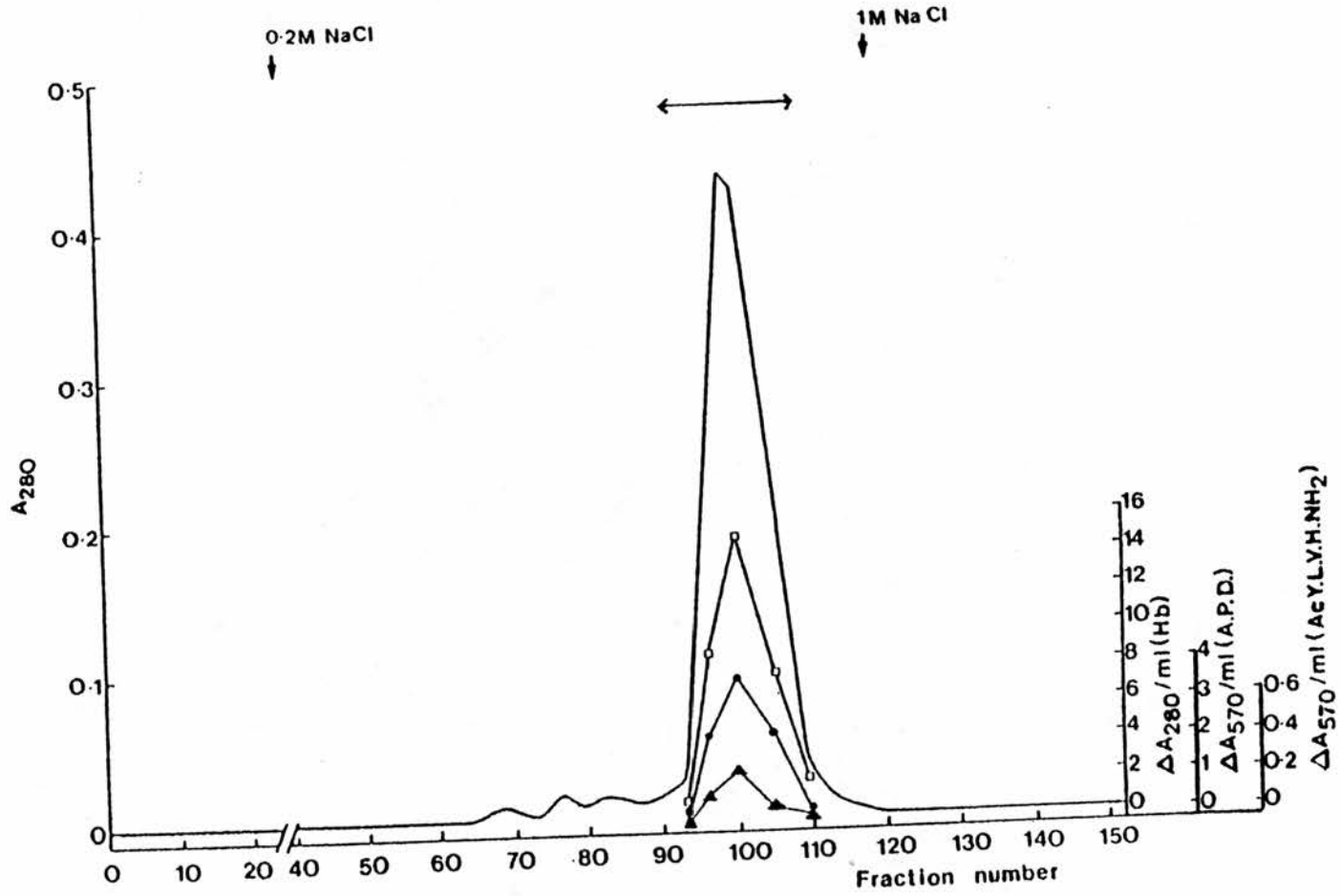

Fig. 13 Rechromatography of peak III on DEAE-Sephadex A50, conditions as in Fig.2. Arrows indicate introduction of NaCl gradients. Fractions were pooled as shown. Symbols as in Fig.11.

it would be surprising if sufficient contaminating proteins to explain this peptidase activity co-chromatographed with peak I in the re-chromatographic step. Furthermore, the specific activity against A.P.D. is constant across the peak, indicating that the sample is pure.

3.5.3 Rechromatography of peak II

For peak II, about 14 mg of freeze-dried material obtained from the chromatography shown in Figure 4 was used. The elution profile is shown in Figure 12, in which the fractions which were pooled are shown. There is clear evidence of contamination by both peaks I and III, and by two minor peaks in the approximate position of peak Ia. It is especially interesting to see that the activity profile for the assay with Ac.Y.L.V.H.NH_2 falls steadily across the peak. This suggests that the activity is actually due to contaminating material, probably peak I. It should be noted that the rechromatography of peak II was performed on three separate occasions, using different samples, and this effect was observed in each case. It thus appears that peak II may have no activity against this substrate.

3.5.4 Rechromatography of peak III

For peak III, 18·9 mg of freeze-dried material obtained from the chromatogram shown in Figure 4 was rechromatographed. The elution profile is shown in Figure 13, from which it may be seen that the peak III isolated from the original chromatogram contained relatively small levels of peaks Ia, I and II but was apparently free from peaks IV-VI. It appears that peak III has activity/...

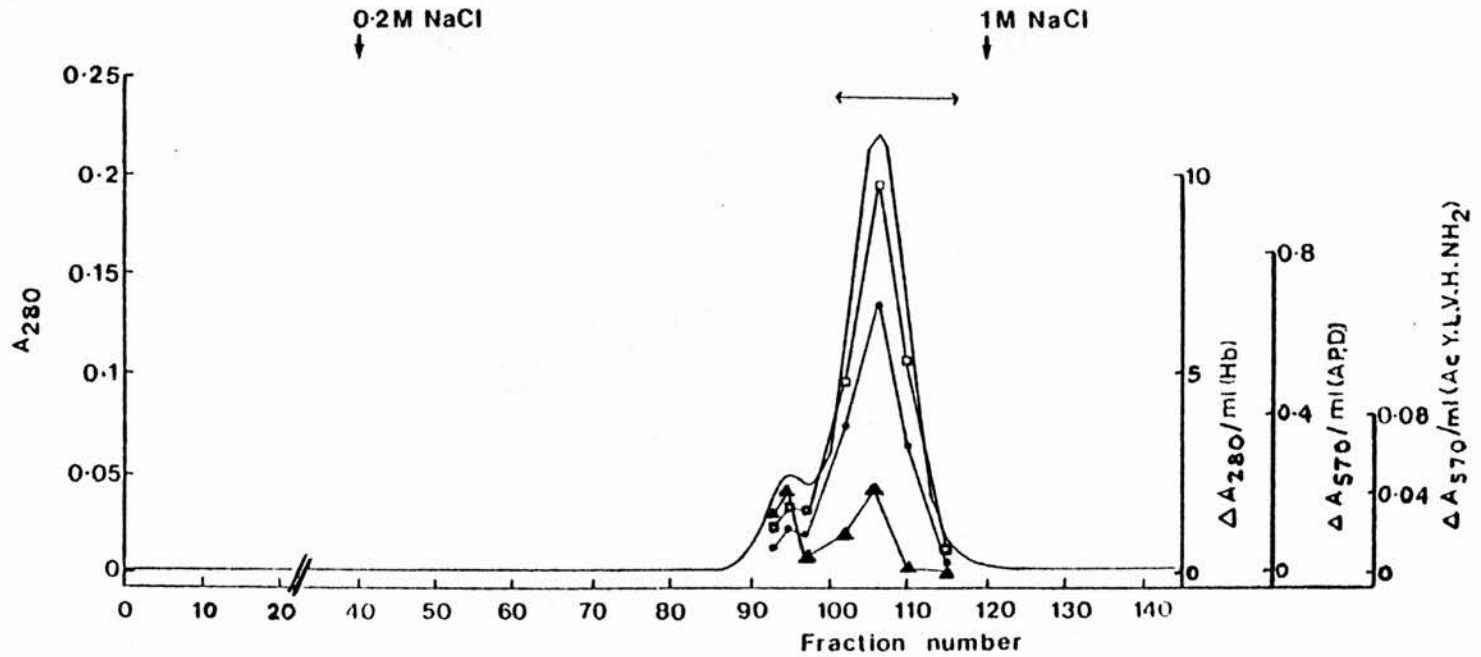

Fig. 14 Rechromatography of peak IV of DEAE-Sephadex A50, conditions as in Fig2. Arrows indicate introduction of NaCl gradients. Fractions were pooled as shown. Symbols as in Fig.11.

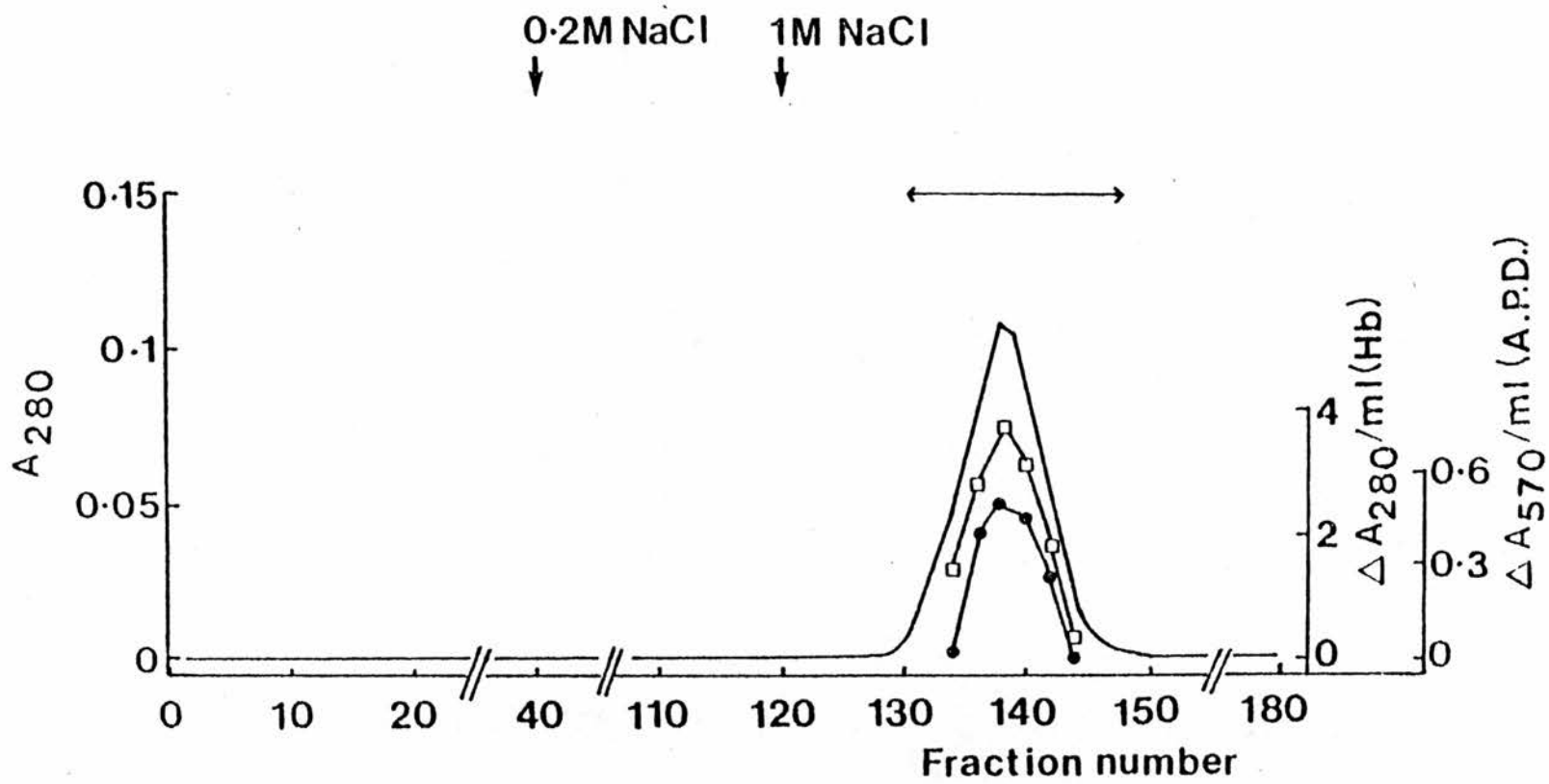

Fig. 15 Rechromatography of peak V on DEAE-Sephadex A50, conditions as in Fig 2. Arrows indicate introduction of NaCl gradients. Fractions were pooled as shown. Symbols as in Fig.11.

activity against all three substrates; specific activities are shown in Table 5. Fractions which were pooled, dialysed and freeze-dried are shown in the diagram.

3.5.5 Rechromatography of peak IV

For peak IV 14·5 mg of freeze-dried material, taken from more than one chromatography of gastric juice, was rechromatographed. Figure 14 shows that the peak IV contains considerable amounts of peak III, which might be expected given the relatively poor separation found in the chromatography of gastric juice (Figure 4). The profile of activity follows the protein profile for all three substrates, so it appears that these activities are probably a genuine property of peak IV.

3.5.6 Rechromatography of peak V

Very little peak V was obtained from any given chromatography of gastric juice, and therefore freeze-dried material from several such chromatographies was pooled, and about 7 mg of this was rechromatographed. Figure 15 shows the elution profile; it may be seen that there was no obvious contamination from any other peak. Fractions were pooled as shown. This material was assayed against haemoglobin and A.P.D. but, because of the very low protein concentration, activity against Ac.Y.L.V.H.NH_2 was not determined. Specific activities are shown in Table 5.

3.5.7 Rechromatography of peak VI

Similarly to peak V, very little of this material was obtained. Peak VI obtained from several chromatographies of gastric juice was pooled/...

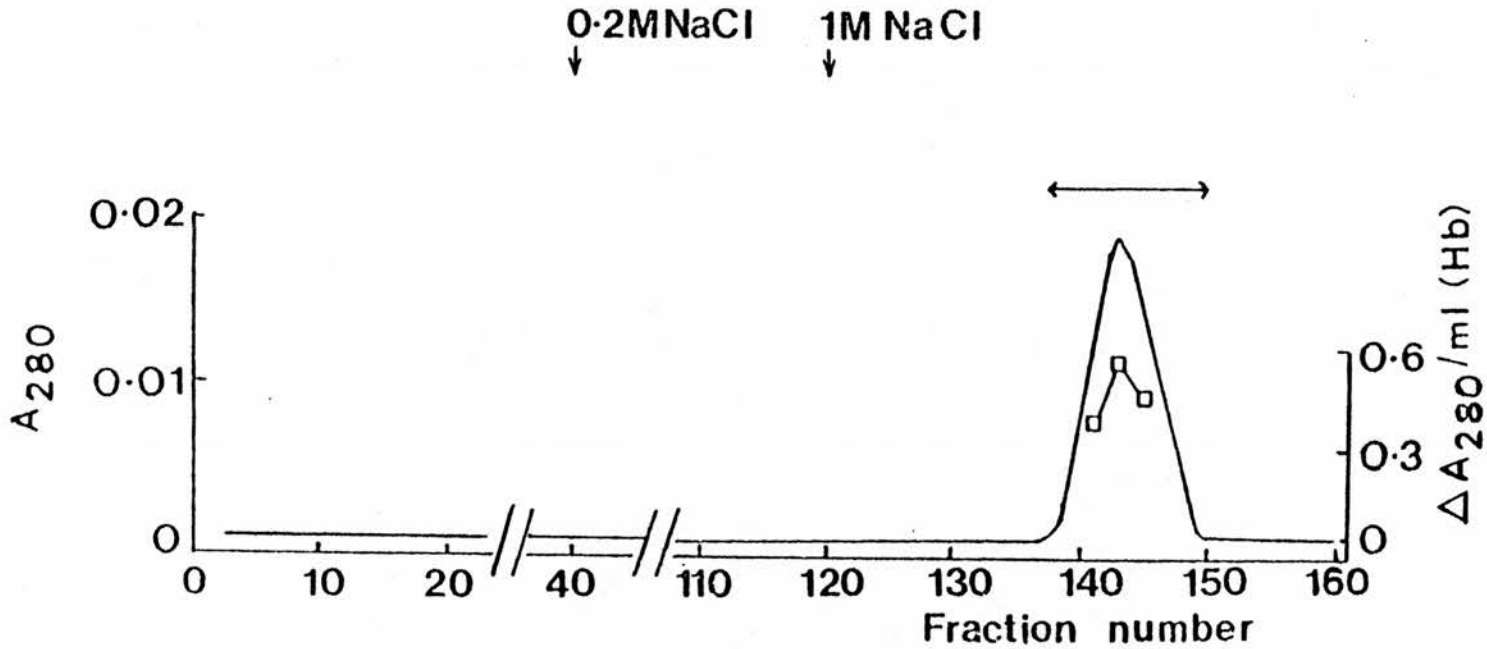

Fig. 16 Reohromatography of peak VI on DEAE-Sephadex A50, conditions as in Fig. 2. Arrows indicate introduction of NaCl gradients. Fractions were pooled as shown. Symbols as in Fig 11.

Table 5 Specific activities of peaks obtained from rechromatography on DEAE-Sephadex A50.

Peak	Haemoglobin	A.P.D.	Ac.Y.L.V.H.NH_2
Ia	0	0	0
I	96	0·9	14·5
II	67	12·9	see text
III	33·7	8	0·47
IV	43·2	3·2	0·22
V	34·5	4·5	N.D.
VI	45·8	N.D.	N.D.

N.D. = not determined.

pooled and about 3 mg of this was used in the rechromatography. The elution profile is shown in Figure 16, from which it appears that this preparation is quite free from other peaks. Fractions which were pooled are shown. There was so little protein present that only assay with haemoglobin was practicable.

Great difficulty was encountered in recovering material from peaks V and VI, especially the latter. Samples of peak VI were often totally destroyed by freeze-drying, possibly as a result of their very low concentration. This occasionally, although not always, occurred with peak V also. An attempt was made to recover peak VI by ammonium sulphate precipitation. Ammonium sulphate was gradually added to a solution of peak VI in an ice bath up to approximately 80% saturation. The solution was left for two days but there was no precipitation. It was thus necessary to revert to freeze-drying to recover any peak VI at all.

A summary of specific activities is given in Table 5 . Some of these may be directly compared to data in Table 2. Thus, e.g. for peak I increases in specific activity against haemoglobin and Ac.Y.L.V.H.NH_2 and a decrease in specific activity against A.P.D. may indicate increased purity. The values shown in Table 5 for peaks IV, V and VI are not strictly comparable with the corresponding data in Table 2 because for these peaks the material used for rechromatography was obtained from more than one chromatogram of gastric juice, and not solely from the chromatogram shown in Figure 4.

3.6 Electrophoresis in agar gels

In order to correlate the samples obtained by chromatography of gastric/....

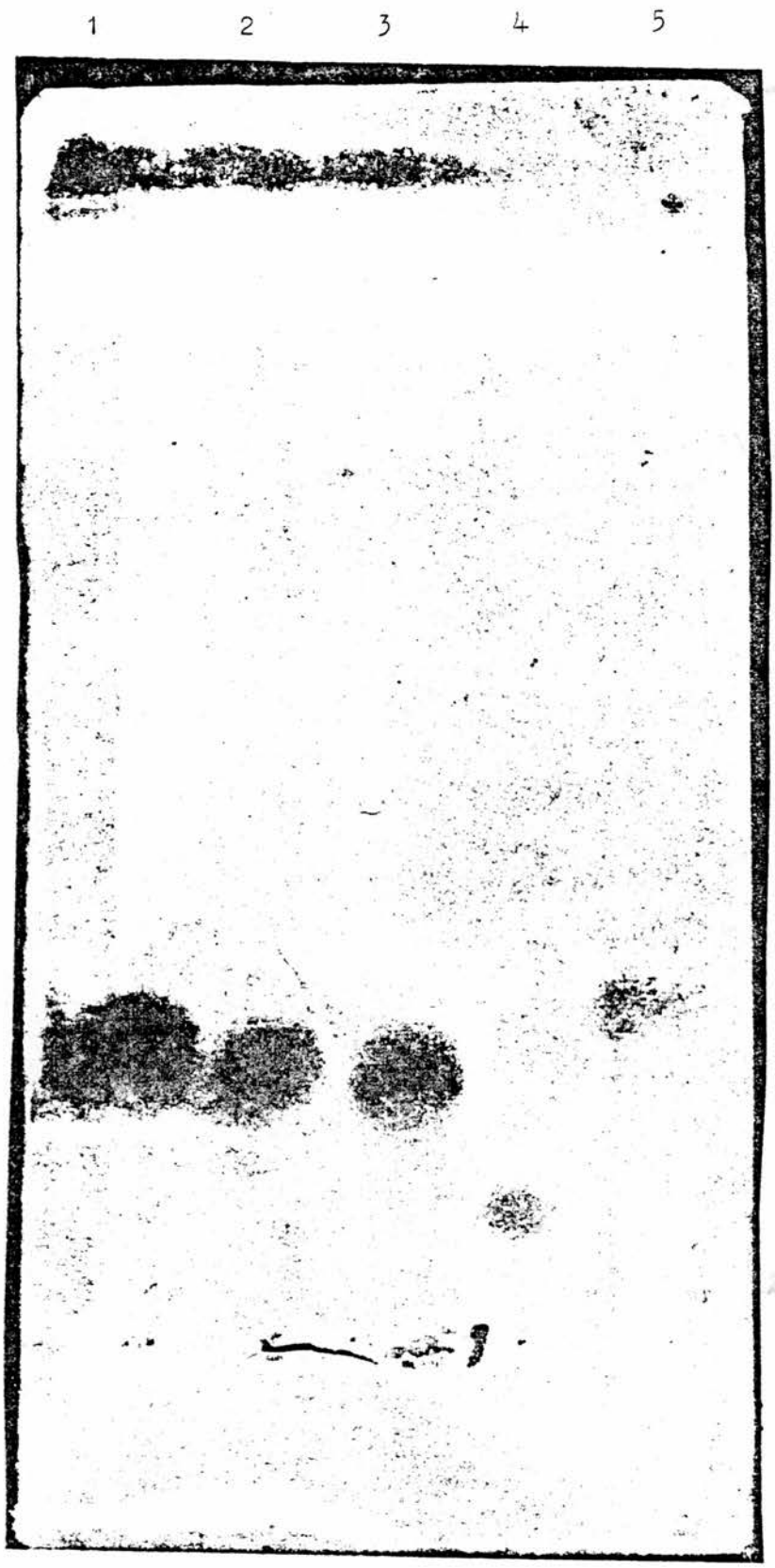

Plate 2. Agar gel electrophoresis of various pepsins at pH 5.3. Electrophoresis was conducted at 10 v/cm length, 3 hours.

Slot 1 - peak IV; slot 2 - peak III; slot 3 - peak II; slot 4 - peak I; slot 5 - pig pepsin A. The gel was stained for activity.

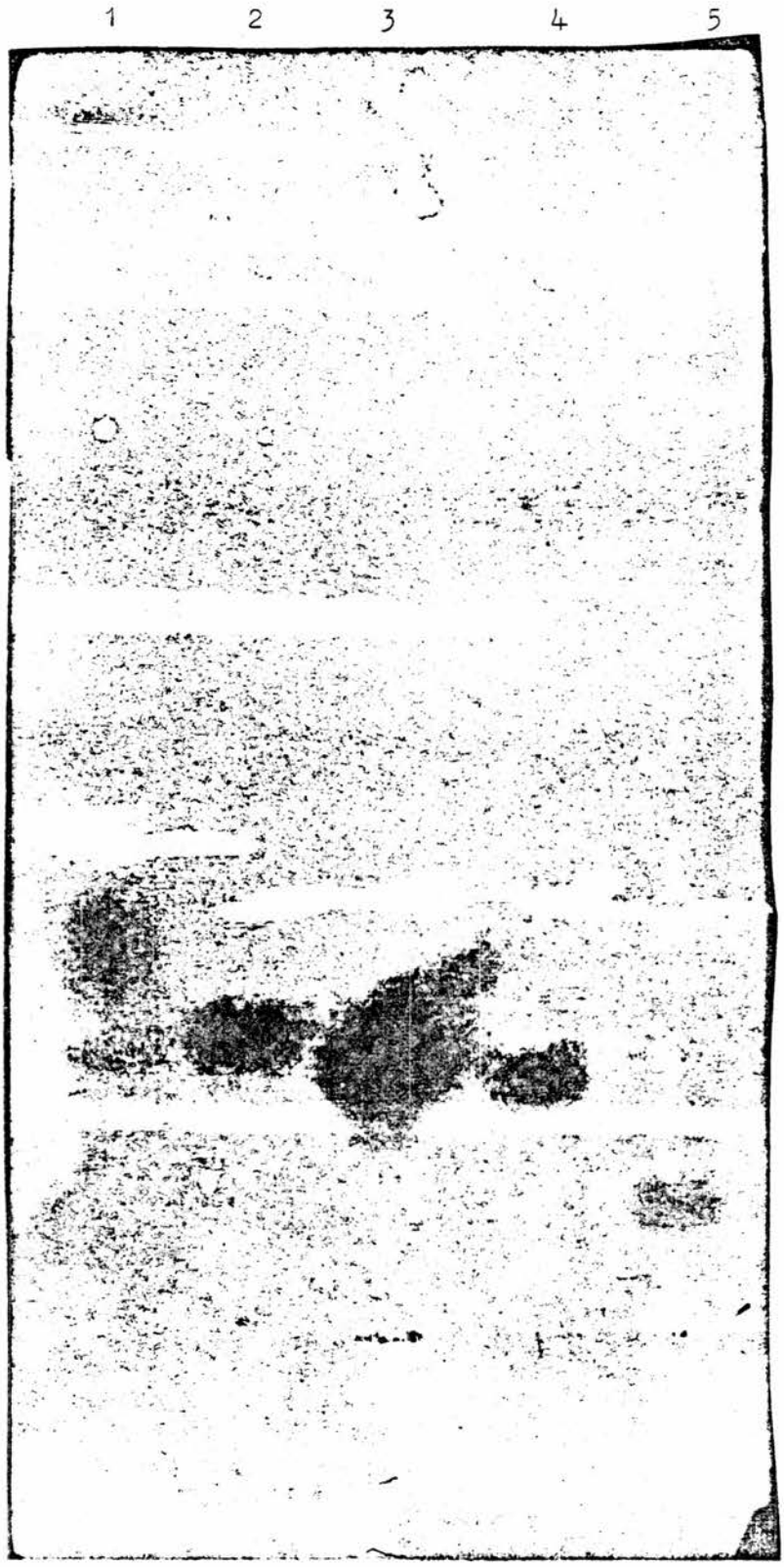

Plate 3. Agar gel electrophoresis of human pepsins at pH 5·3. Electrophoresis was conducted at 10 v/cm length, 3 hours.
Slot 1 - peak V; slot 2 - peak IV; slot 3 - peak III; slot 4 - peak II; slot 5 - peak I. The gel was stained for activity.

Table 6 Relative mobilities in agar gel electrophoresis of pepsins compared to that of pig pepsin.

Pepsin [1]	Relative mobility	Pepsin [2]	Relative mobility
pig	1		
I	0·63	5	0·54
II	0·9		
III	0·91	3	0·90
IV	0·95	3a	0·99
V	1·11	2	1·10

[1] reported here

[2] Roberts and Taylor (1978a)

of gastric juice on DEAE-Sephadex A50 with pepsins studied by Taylor and coworkers, agar gel electrophoresis was performed in Dr. Taylor's laboratory. At the time of doing these experiments no sample of peak VI was available. Plates 2 and 3 show the results obtained. The samples used, except for pig pepsin, were isolated following second chromatography on DEAE-Sephadex A50. The method of electrophoresis is described in Sect. 2.8.2. Pepsins were made up to 0·1 mg/ml in 1 mM HCl except for the pig pepsin which was 0·02 mg/ml. Sufficient of each sample was applied to fill the 10 x 1 mm slots without overflowing. Bromophenol blue marker was not added to samples, but was placed in separate slots at either side of the sample-containing slots. Electrophoresis was conducted at constant voltage, 10 v/cm for three hours. Plate 2 shows the mobilities of peaks I-IV relative to that of the pig pepsin marker. The pig pepsin runs slightly ahead of peaks II-IV, as was found in poly-acrylamide gel electrophoresis (Plate 1). From this gel (Plate 2) it is clear that peak I is equivalent to pepsin 5 (Taylor's nomenclature), while peak III is equivalent to pepsin 3.

Plate 3 shows the mobilities of peaksI-V. Peaks II-IV have very similar mobilities, as they do in polyacrylamide gels (see Plate 1). It seems that peak V is probably equivalent to pepsin 2 reported by Taylor.

Mobilities of these samples relative to that of pig pepsin are shown in Table 6. Comparison of these mobilities with those reported by Roberts and Taylor (1978a) helps to confirm the correlation. It appears that peak IV reported here may be equivalent to pepsin 3a. This was suggested tentatively in Sect. 3.3.5, on the basis of the order/...

order of elution of the peaks from ion-exchange columns. Although peak V was not actually electrophoresed in the same gel as the pig pepsin marker, it is probably valid to estimate its relative mobility using data from both Plates 2 and 3. As may be seen from Table 6, the relative mobility of peak V calculated in this way agrees well with that reported by Etherington and Taylor (1967) for pepsin 2.

3.7 Further purification of proteins

Although electrophoresis of pepsin samples in agar gels, following double chromatography on DEAE-Sephadex A50, indicated that most samples appeared to be quite free from contamination by other pepsins, it was possible that during the three-day period of chromatography autodigestion occurred with consequent production of inactive material, previous experiments having indicated that autolysis did not produce enzymically-active products. Thus, it was thought that a relatively short gel filtration step might be useful in removing any such material.

3.7.1. Gel filtration

The method used for all proteins was as follows. A sample of protein which was two-fold chromatographed as described in Sect. 3.5 and freeze-dried was dissolved in the smallest possible volume of 0·1 M acetate buffer pH 4·0 and centrifuged as described in Sect. 2.7. The supernatant was applied to a 2·5 cm x 40 cm column of Sephadex G 75, prepared as described in Sect. 2.7. The chromatogram was developed as detailed in that section. Flow-rate was maintained at 20 ml/hr and fractions of 5 ml were collected.

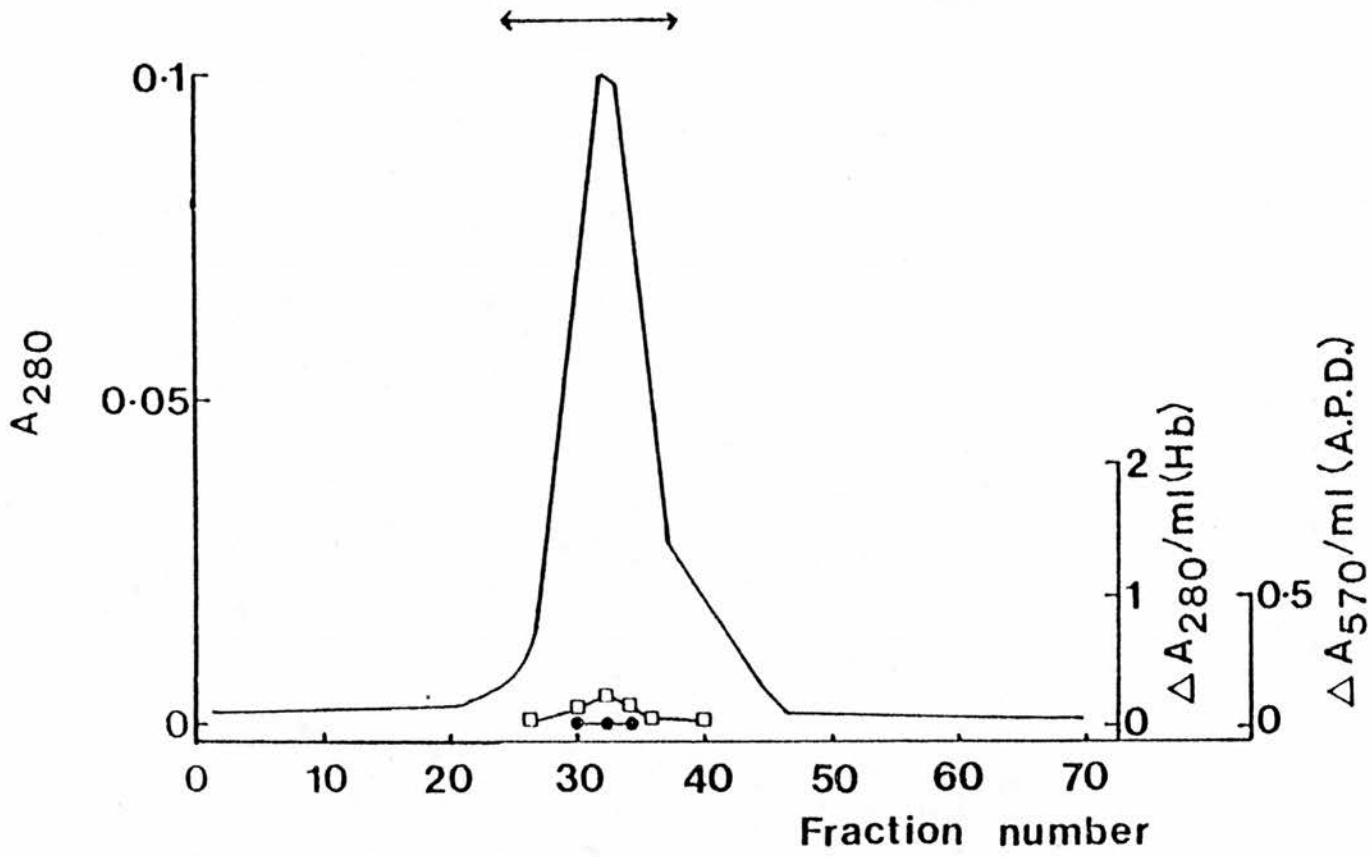

Fig. 17 Gel filtration of peak Ia on Sephadex G75, 0·1 M acetate buffer, pH 4·0. Column 2·5 cm x 40 cm; flow-rate 20 ml/hr. Fractions (5 ml) were pooled as shown. — , protein; □, haemoglobin assay; ●, A.P.D. assay.

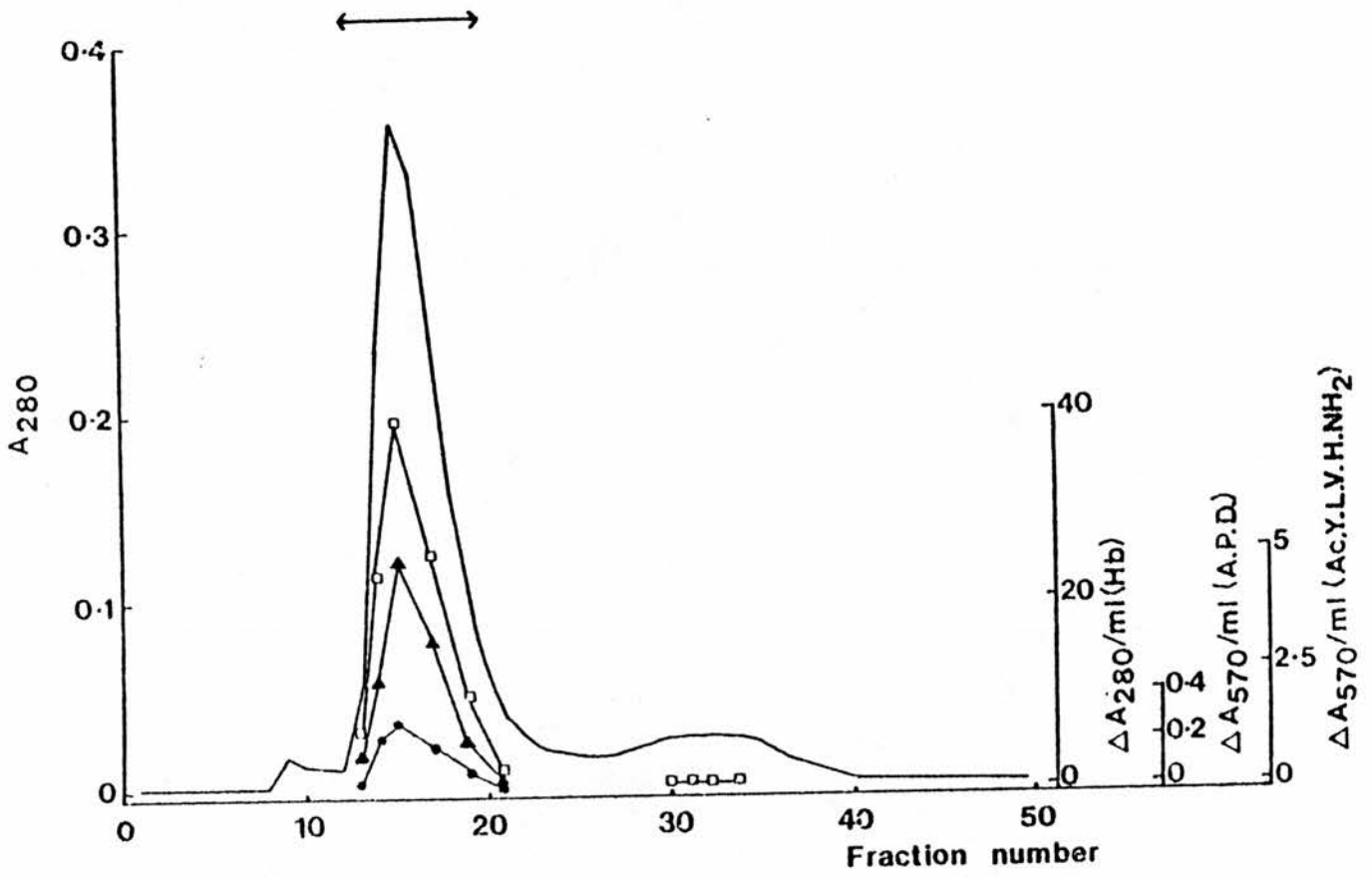

Fig. 18 Gel filtration of peak I on Sephadex G75, conditions as in Fig.17. Fractions were pooled as shown. — , protein; □, haemoglobin assay; ▲, Ac.Y.L.V.H.NH_2 assay.

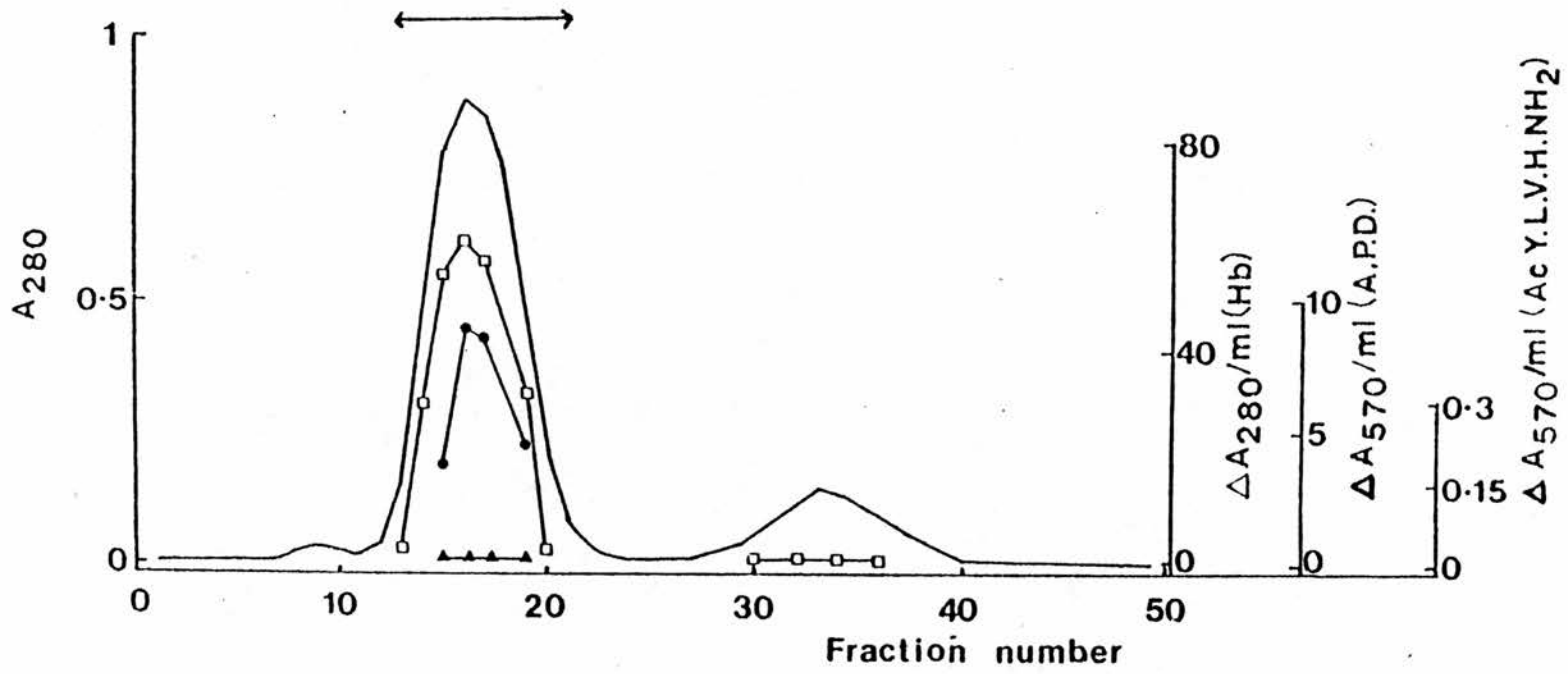

Fig. 19 Gel filtration of peak II on Sephadex G75, conditions as in Fig 17. Fractions were pooled as shown. Symbols as in Fig. 18.

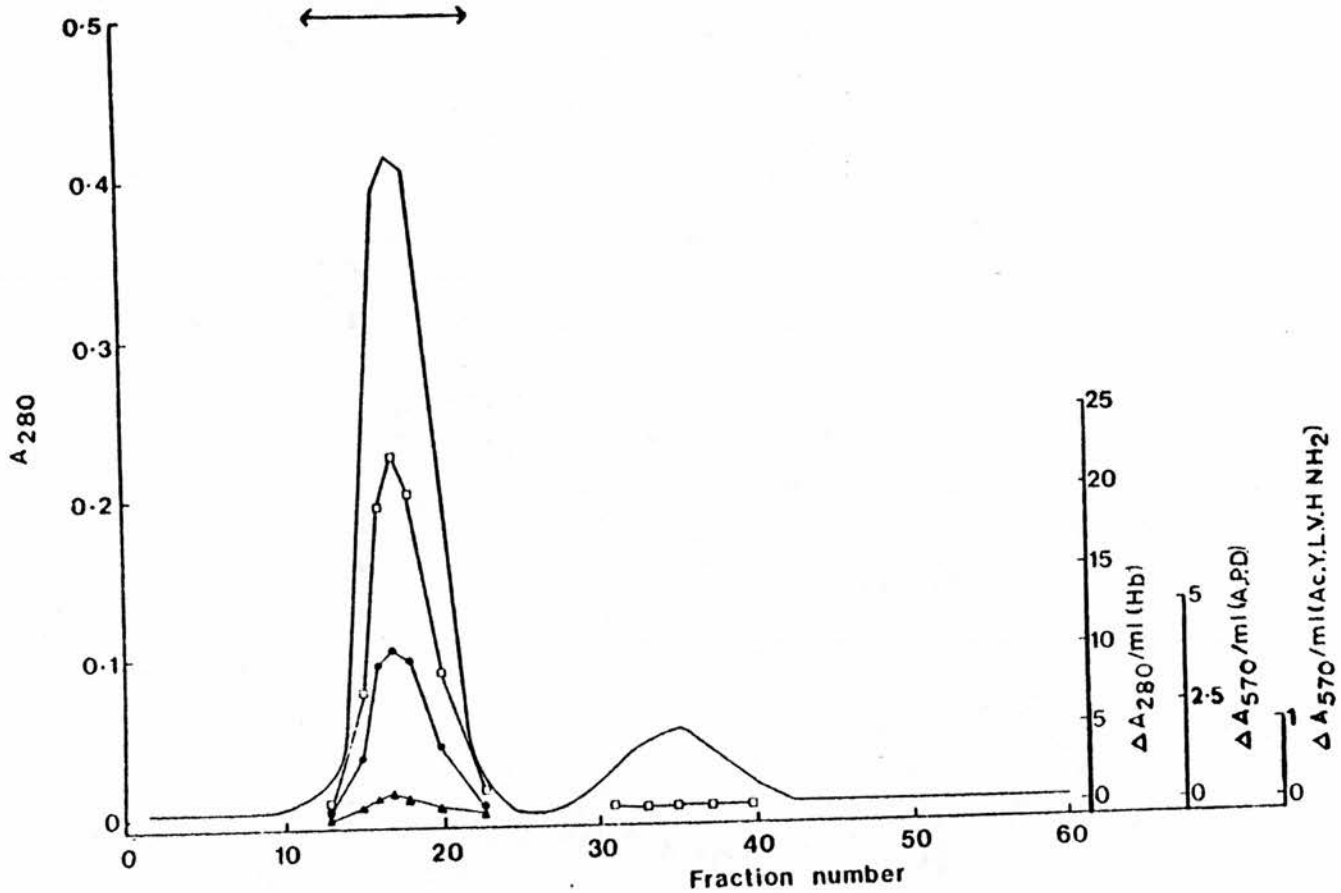

Fig. 20 Gel filtration of peak III on Sephadex G75, conditions as in Fig. 17. Fractions were pooled as shown. Symbols as in Fig. 18.

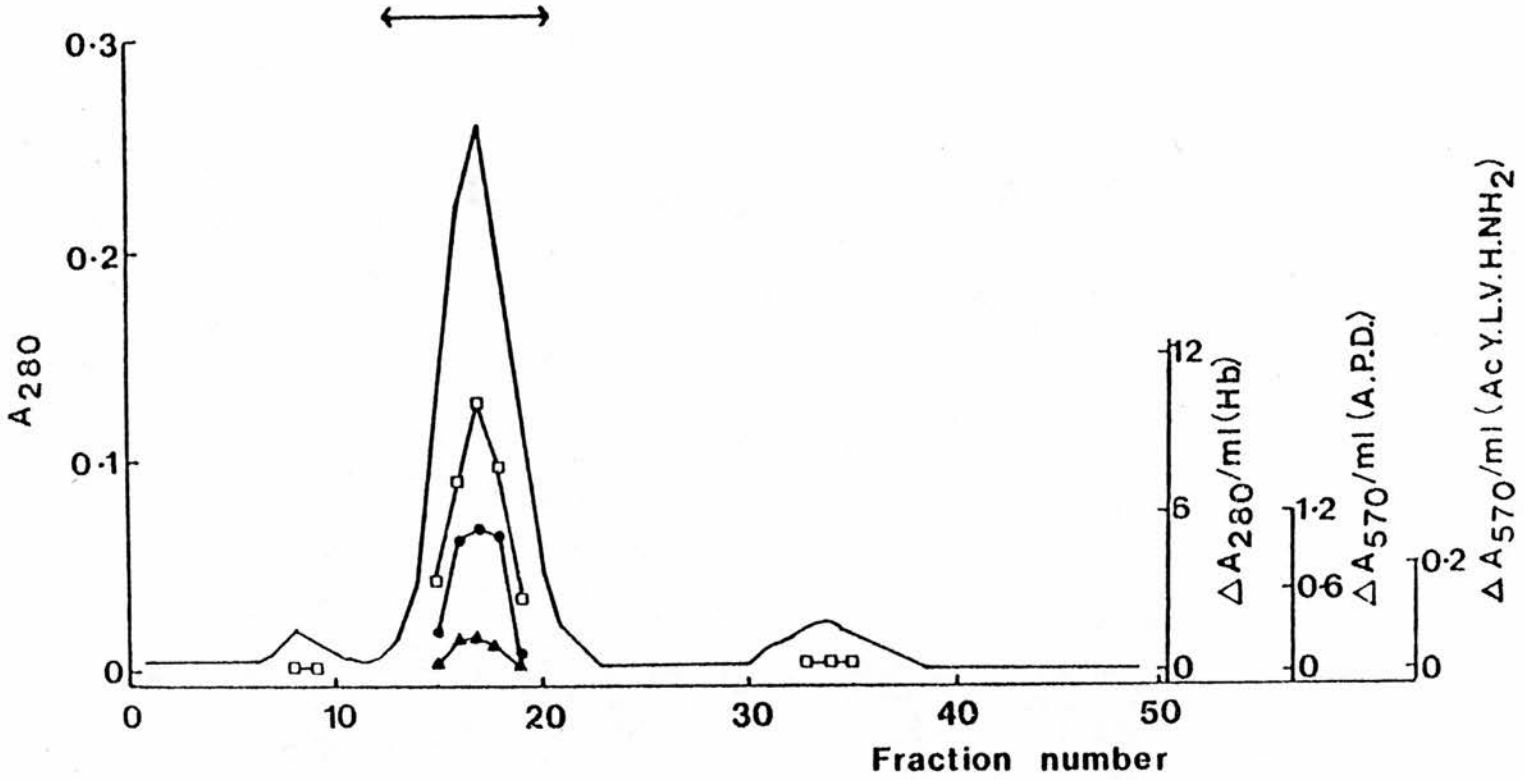

Fig 21 Gel filtration of peak IV on Sephadex G75, conditions as in Fig. 17. Fractions were pooled as shown. Symbols as in Fig. 18.

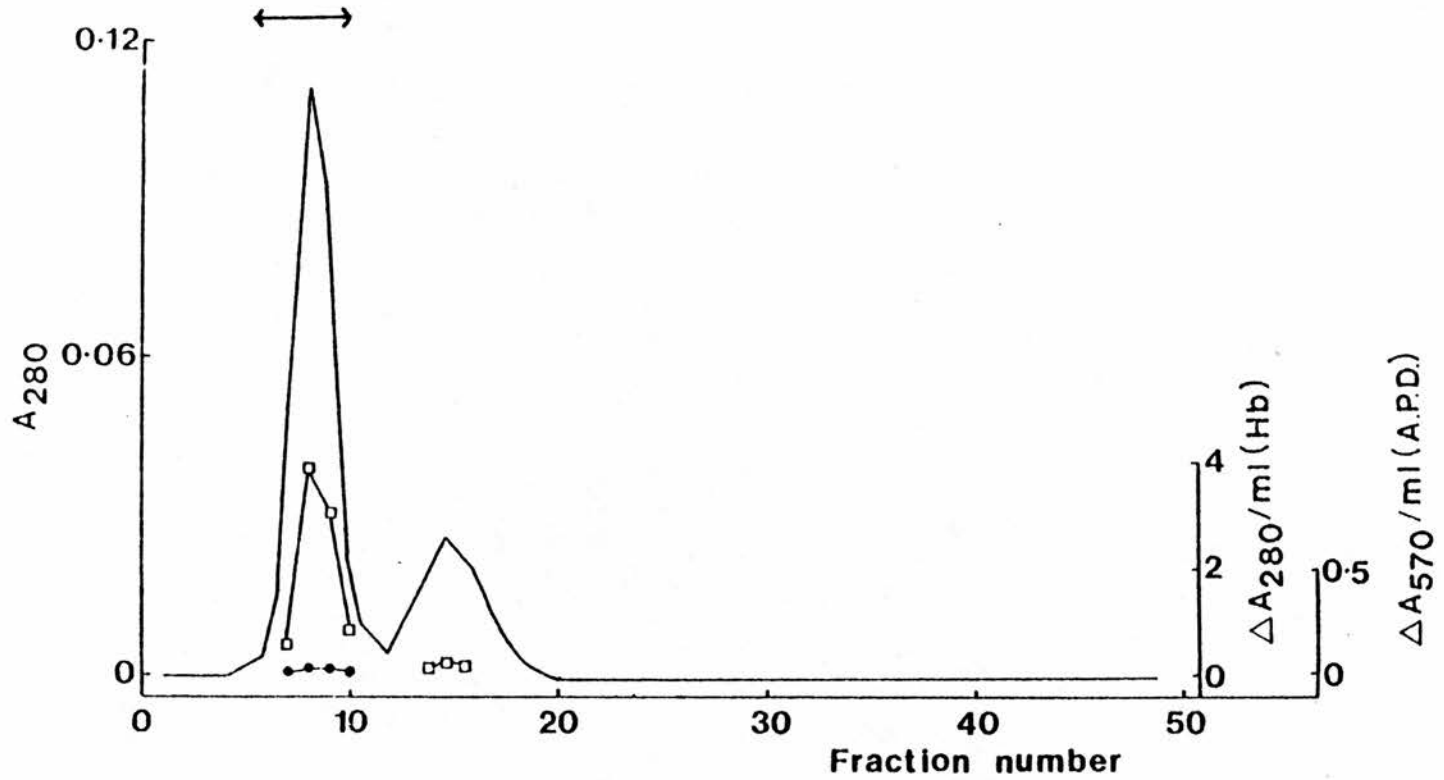

Fig. 22 Gel filtration of peak V on Sephadex G75, conditions as in Fig.17. Fractions were pooled as shown. Symbols as in Fig. 18.

Table 7 Specific activities of proteins after gel filtration.

Peak	Haemoglobin	A.P.D.	Ac.Y.L.V.H.NH_2
Ia	3	0	N.D.
I	108·5	0·74	14·3
II	69	7·8	0
III	52·4	10·7	0·6
IV	38·5	3·9	0·2
V	35	2	N.D.

N.D. = not determined.

Figures 17-22 show the elution profiles for these gel filtrations and the specific activities, where determined, are given in Table 7. Comparison of ratios of specific activities in Tables 2,5 and 7 suggest that there may not be enough precision in these assays to distinguish peaks II,III and IV; careful analysis of the precision and reproducibility of the assays would be required.

For peak Ia, about 5 mg of freeze-dried material obtained from the second chromatography on DEAE-Sephadex A50 was used. It may be seen from Figure 17 that peak Ia is virtually inactive against haemoglobin and A.P.D. Also, the peak is not symmetrical having a slight shoulder, perhaps representing contamination from another peak. SDS-polyacrylamide gel electrophoresis (Sect. 3.9) indicated that the molecular weight of peak Ia is about 45,000. From the position of its elution from Sephadex G75, this material appears to have a much lower molecular weight. It may be that peak Ia is abnormally retarded on Sephadex G75, perhaps suggesting that it is a proteoglycan.

Peak I clearly shows activity against all three substrates (Figure 18); 12 mg of freeze-dried material was used. A relatively small peak appeared before the main peak; the nature of this material, which was not assayed against any of the three substrates, is not known. A considerable amount of contaminating material, apparently of lower molecular weight than peak I and inactive against haemoglobin, was also detected.

Figure 19 shows that peak II was inactive against Ac.Y.L.V.H. NH_2, as was suggested during chromatography on DEAE-Sephadex A50. Peak II appears to be active against haemoglobin and A.P.D.

From Figures 20 and 21, peaks III and IV appear to be active against all three substrates, although their specific activities are quite distinct from each other. As for peak I, Figure 21 shows that a small peak appeared in the chromatogram of peak IV, this material being eluted before the main peak. In both the diagrams, small peaks of inactive material, eluted after the main peaks and therefore presumably of lower molecular weight, are clearly visible.

Very/...

1 2 3 4 5 6

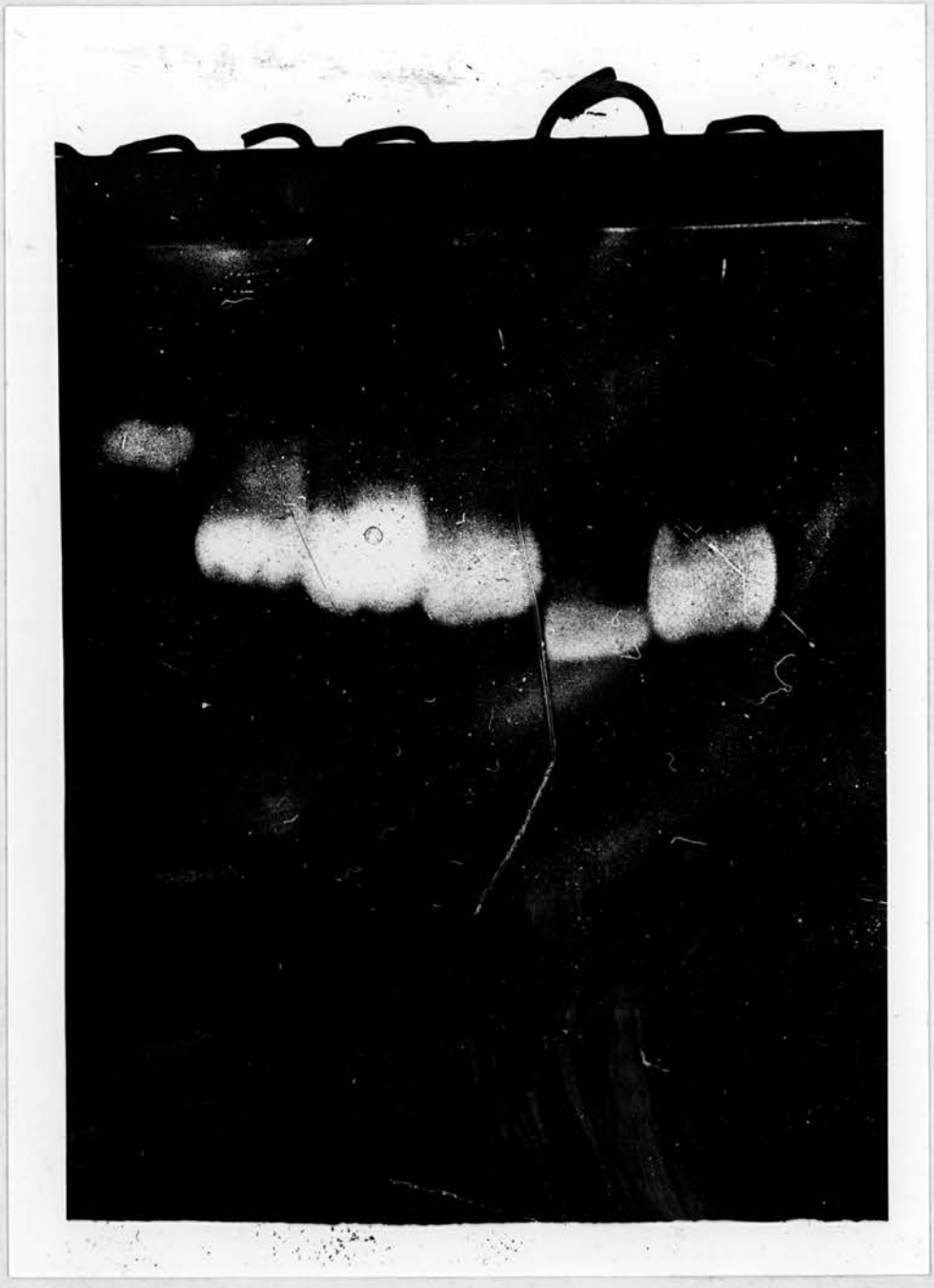

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Plate 4. Electrophoresis of pepsins at pH 4·0 in 8% acrylamide gel. Pepsins were obtained by double chromatography on DEAE-Sephadex A50 and gel filtration on Sephadex G75. Electrophoresis was conducted at 150 v for 4 hours. The gel was stained for activity.

slot	1	2	3	4	5	6
sample	I	II	III	IV	V	VI

Very little peak V was available for gel filtration and it may be seen from Figure 22 that the A_{280} of fractions was extremely low. The absorbancies at 570 nm measured in the A.P.D. assay for peak V were so low that they may not be wholly reliable. Figure 22 shows peak V emerging before the void volume of the column. This is impossible; therefore it would seem likely that a number of fractions must have been mistakenly discarded.

Inactive material was detected in some of the gel filtration experiments. Although the nature of this material was not investigated further, it may be that the material which appeared after the major peaks represents autolytic breakdown products. Very little, if any, of this appears in Figure 17, the gel filtration of peak Ia. However, since peak Ia emerges near the end of the chromatogram, any low molecular weight products would not be apparent; also, peak Ia seems to be enzymically inactive, so autolysis would not occur. Presumably any material of a higher molecular weight than that of the major peaks, as can be seen in Figures 18 and 20, are not degradation products.

For reasons already explained (Sect.3.5.7) it was not possible to obtain sufficient peak VI for gel filtration to be worthwhile.

3.8 Electrophoresis in polyacrylamide gels.

Following purification by gel filtration, freeze-dried samples were subjected to electrophoresis in polyacrylamide gels. A typical example of such a gel is shown in Plate 4. In this case a stacking gel was used as described in Sect. 2.8.1, with β-alanine/HCl buffers. Samples/...

Table 8 Relative electrophoretic mobility, in polyacrylamide gels, of pepsins obtained by gel filtration.

Pepsin	Relative mobility
I	0·60
II	0·83
III	0·90
IV	0·93
V	1
VI	0·96

Samples were prepared as previously described (Sect.2.8.1) and 50 µl of each was applied to the gel. Electrophoresis was conducted at constant voltage, 150 v for four hours.

It was particularly important at this stage to establish whether or not samples were pure, so increased volumes of sample were applied. It is quite clear that peak I is, as far as can be seen, free from other pepsins. Similarly it seems that peaks II-VI are free from peak I. There does appear to be some faint trailing in the tracks containing peaks II and III but there does not seem to be any distinct band of peak I in these tracks. Thus, as far as can be judged, it appears that peaks I-V are quite free from other haemoglobin-digesting material. However, because peaks II-IV have very similar mobilities, and the bands overlap slightly especially bands II and III, it is not certain that these peaks do not cross-contaminate one another.

The sample of peak VI, which was not purified by gel filtration, has produced a fairly broad band without any clearly distinct band of any other enzyme. Because band VI is broad it is not possible to say definitely that it is free from the other faster bands. Chromatographically peak VI appears to be free from such material, as shown in Figure 16.

Table 8 shows the relative mobilities of the proteins, compared to peak V, which was previously found to have the same mobility as a pig pepsin marker (Table 4). The relative mobilities of proteins in Plates 1 and 4 show good agreement, as may be seen by comparison of Tables 4 and 8.

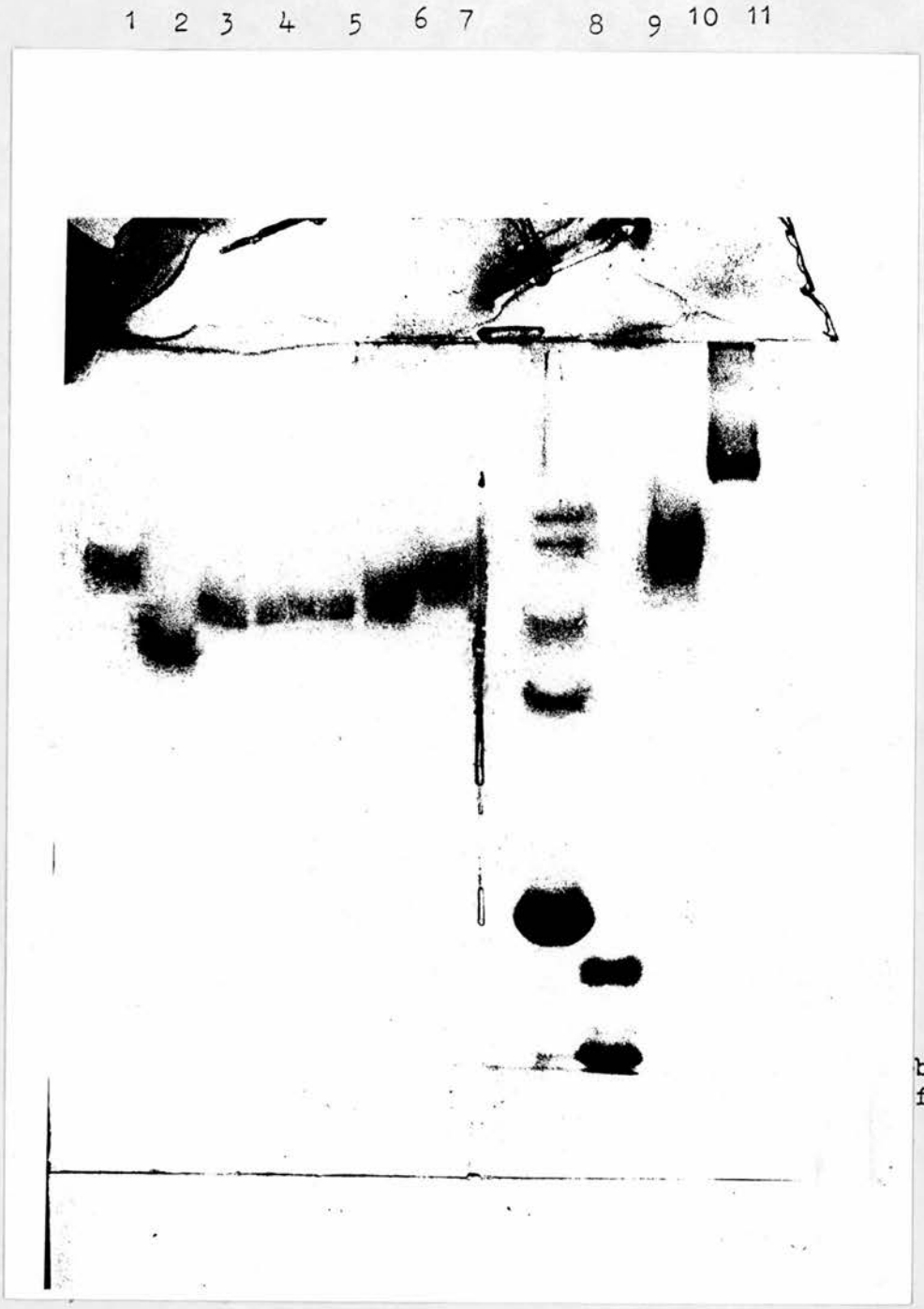


Plate 5 SDS-polyacrylamide gel electrophoresis. The gel was 14% acrylamide. Electrophoresis was conducted at 50 v for 15 hours.

slot 1, peak Ia; slot 2, peak I; slot 3, peak II; slot 4, peak III; slot 5, peak IV; slot 6, peakV; slot 7, peak VI; slot 8, myoglobin; slot 9, chymotrypsin; slot 10, ovalbumin; slot 11, bovine serum albumin.

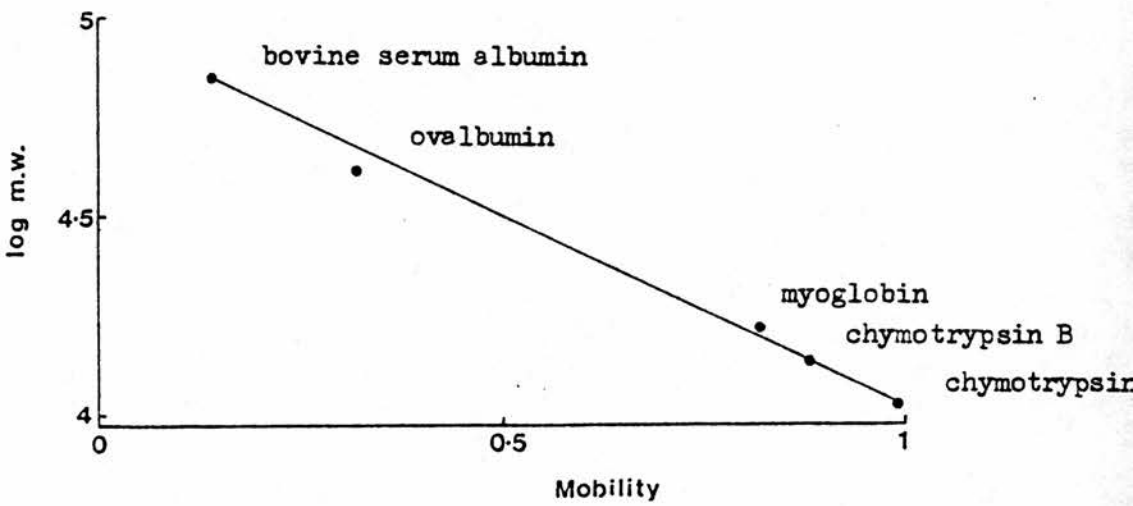


Fig. 23 A plot of $\log_{10}$ molecular weight of various standard proteins as a function of electrophoretic mobility. Electrophoresis was conducted at 50 v for 15 hours in an SDS-polyacrylamide gel of 14% acrylamide.

Table 9 Molecular weights determined by electrophoresis in SDS-polyacrylamide gels.

Protein[1]	Molecular weight	Pepsin[2]	Molecular weight
Ia	45,000		
I	35,000	5	34,600
II	39,800		
III	39,800	3	37,200
IV	40,000		
V	40,000	2	39,950
VI	41,000	1	43,800

[1] reported in this work

[2] from Roberts & Taylor (1972)

3.9 Molecular weights of the proteins

Molecular weights of the various proteins were determined using an SDS-polyacrylamide gel, prepared, electrophoresed and stained as described in Sect.2.8.1. It has been reported that pepsin binds very weakly to SDS (Nelson 1971). However, in that paper, the conditions were selected such that pepsin retained full activity, binding being studied at pH5. There is little reason to suspect that denatured pepsin will behave in this way.

The standard proteins used were myoglobin (molecular weight 17,000), chymotrypsin (molecularweight chymotrypsin B 14,000, chymotrypsin C 11,000), ovalbumin (molecular weight 43,000) and bovine serum albumin (molecular weight 68,000). Samples of these standards and of the proteins being analysed were made up such that after addition of dissociation buffer, mercaptoethanol and bromophenol blue, the final concentrations were 2 mg/ml, and 50 µl of each test sample and 20 µl of each standard was applied to the gel. Electrophoresis was performed at constant voltage, 50 v for 15 hours.

It was found that, using gels of 8% and 10% acrylamide, myoglobin and chymotrypsins B and C ran with the bromophenol blue front. Plate 5 shows a gel of 14% acrylamide. Figure 23 represents the standard graph obtained, using a plot of log molecular weight v. mobility. Mobilities are calculated relative to the distance moved by the bromophenol blue. Table 9 shows the molecular weights determined for the proteins being analysed, compared with results published by Taylor. It can be seen that these molecular weights show/...

show generally good agreement. It has been suggested that SDS-polyacrylamide gels generally give results within about 10% of the actual molecular weight, glycoproteins especially tending to give anomalous results (Allen 1981). Thus, it may be best to regard such results as apparent molecular weights until confirmed by other evidence.

It is interesting to note that, under the reducing conditions of an SDS-polyacrylamide gel, the proteins obtained in this work appear to be free from other proteins; the fact that the bands V and VI are rather broad may reflect some degree of heterogeneity, or possibly overloading although the amount of these peaks used was the same as for the other samples. However, because peaks II-VI have very similar mobilities this technique does not really offer proof that they are free from each other. It does appear that these pepsins are free from peak I, and vice-versa.

Table 10 Amino acid analyses, shown as residues per molecule to the nearest integral number from single analyses, for peaks III and I compared with those for pepsin and gastricsin.

	Pepsin[1]	III	Gastricsin[1]	I
½-cys	6	6	6	5
asx	40	38	26	28
thr	27	26	21	18
ser	43	41	32	31
glx	31	35	39	42
pro	19	19	17	32
gly	35	36	33	28
ala	18	20	18	17
val	27	29	23	22
met	5	6	5	4
ile	25	24	13	8
leu	22	24	25	29
tyr	15	18	17	14
phe	15	16	15	19
his	1	1	1	1
lys	0	1	0	3
arg	3	3	3	3

[1] Mills, J.N., Tang, J. (1967)

Table 11 Amino acid analyses of several proteins, given as residues per molecule to the nearest integral numbers, from single analyses.

	Ia	II	IV	V	VI
½-cys	6	5	6	2	2
asx	57	37	42	33	35
thr	34	27	30	35	50
ser	34	41	44	36	42
glx	61	33	37	29	27
pro	18	28	15	10	4
gly	31	36	38	28	33
ala	20	21	21	18	22
val	26	30	26	19	23
met	2	6	6	5	7
ile	6	20	22	14	17
leu	70	27	28	66	77
tyr	5	18	17	14	5
phe	13	17	17	32	31
his	6	1	1	4	1
lys	10	2	1	2	5
arg	7	4	3	4	5

3.10 Amino Acid Analyses

Proteins obtained by double chromatography on DEAE-Sephadex A50 and gel filtration as described, and shown to be pure by polyacrylamide gel electrophoresis as in Plate 5, were hydrolysed and prepared for amino acid analysis as detailed in Sect. 2.9. The results of single analyses are given in Tables 10 and 11. The analyses presented by the present author are based on the molecular weights shown in Table 9.

Some general points concerning the amino acid analyses can be made. Acid hydrolysis at 110° for 24 hours tends to cause some loss of serine, threonine, methionine and tyrosine. In the experiments reported here, thioglycolic acid was used to protect methionine and tyrosine (Sletten et al 1968).

Thioglycolic acid can be used to protect tryptophan during acid hydrolysis, as well as methionine and tyrosine. However, to protect tryptophan a final concentration of 4% thioglycolic acid is necessary (Matsubara and Sasaki 1969). In the analyses reported here, the final concentration of thioglycolic acid was only 0·033%, so it is not possible to estimate the number of tryptophan residues present. However, loss of serine and threonine may have occurred. Moore and Stein (1963) have reported that decomposition of serine and threonine causes the appearance of small peaks in the region of tryptophan, serine, cystine or methionine. Native tryptophan elutes between histidine and lysine (Matsubara and Sasaki 1969)*, and a very small peak was seen in this region in all the chromatograms reported here. This may therefore represent loss of serine and/or threonine. Moore and Stein (1963) suggested that losses of serine and threonine were 10%/...

* However, the elution position of tryptophan depends on buffer programme and analyser.

10% and 5% respectively; it was recommended that these specific correction factors may be used. However, Blackburn (1968) has indicated that degradation kinetics depend on both the particular protein and the experimental conditions. Thus, these correction factors have not been applied here because of this uncertainty. The most accurate method of determining serine and threonine involves hydrolysis of the protein for several different time periods and extrapolation of the data to zero time. Mills and Tang (1967) used this method for pepsin and gastricsin. As may be seen, the values given in Table 10 show reasonable agreement with the published values.

Under the experimental conditions used here it is impossible to distinguish aspartate and glutamate from their respective amides, because the latter are quantitatively hydrolysed to the former. Thus, these residues are shown in Tables 10 and 11 as asx and glx.

Valine and isoleucine may sometimes be released relatively slowly from proteins, perhaps because of steric hindrance of hydrolysis by β -branched sidechains. Thus, if bonds between these amino acids are not hydrolysed completely there may be some error in their reported values. Mills and Tang (1967) found that these values, for pepsin and gastricsin, reached maximum after 40 hours hydrolysis. The values shown in Table 10 for peak III show reasonable agreement with these published data. The estimates of valine and isoleucine for peak I are discussed below.

Cystine and cysteine cannot be distinguished because performic acid oxidation converts both these residues to cysteic acid, in which form they are quantitated.

Table 10 shows the results for peaks I and III compared with those from other workers. Peak I is aligned with gastricsin and peak/...

peak III (the major pepsin) with pepsin, gastricsin and pepsin being the enzymes isolated by chromatography on Amberlite IRC 50. The analyses are in broad agreement for most residues, but a number of points can be made. A notable exception is the value of 32 residues of proline reported here for peak I. In this case, the ratio of absorbance at 440 nm to that at 570 nm for points across the peak is not constant, indicating that there may be some impurity present. Cysteine can interfere with proline (Moore and Stein 1963; Glazer et al 1976). However, in general cysteine and cystine are destroyed by acid hydrolysis, so it seems unlikely that this would explain the anomalous value for proline, especially since the protein only contains 5-6 cystine/cysteine residues per molecule. It is noticeable that the value for proline for peak III given in Table 10 compares well with the published value.

Also the peaks for isoleucine and leucine for peak I were poorly resolved, with a shoulder on the trailing edge of the leucine peak. These figures may therefore be unreliable.

Mills and Tang (1967) found that neither pepsin nor gastricsin contain lysine, whereas the present author found one residue per molecule for peak III and three residues per molecule for peak I. Korbòva et al(1977) reported two lysine residues per molecule for both pepsin and gastricsin.

Table 11 shows the amino acid analyses for the other proteins. It may be seen that peak Ia, which probably is not a pepsin, is relatively high in glutamate/glutamine, aspartate/asparagine, and in basic residues. Also, peak Ia is relatively low in isoleucine and high in leucine. These peaks seem quite symmetrical and thus may be uncontaminated.

Peaks V and VI, which chromatograph close together, have a number of/...

of similarities in their amino acid compositions. It is especially noticeable that only two cystine/cysteine residues per molecule were found in both peaks V and VI. Almost all pepsins, from whatever species, have been reported to contain six such residues per molecule.

Peaks V and VI are shown to be remarkably high in leucine. However, visual inspection of the charts shows that the leucine peaks for these proteins are asymmetrical, suggesting that there may be contaminating material present. The nature of this material is unknown. Peaks V and VI may contain carbohydrate (A.P. Ryle, personal communication). The amino acid analyser will detect amino sugars but not neutral sugars or uronic acids, in a protein hydrolysate. Hexosamines appear between phenylalanine and lysine; glucosamine may co-elute with phenylalanine (Glazer et al 1976). It is noticeable that peaks V and VI contain high values of phenylalanine compared to the other proteins; perhaps these values are distorted by the presence of amino sugars. However the amino sugars are not expected to interfere with leucine. The leucine values for peaks V and VI are remarkably high but the cause is unclear.

It is typical of enzymes of the pepsin-type that the ratio of glutamate: aspartate (including amides) is around 1·0 or less, while that of gastricsin is above 1·0. On this basis, all enzymes reported here except peak I, are of the pepsin-type while peak I is gastricsin-like, using the nomenclature of Richmond et al (1958, see Table 1).

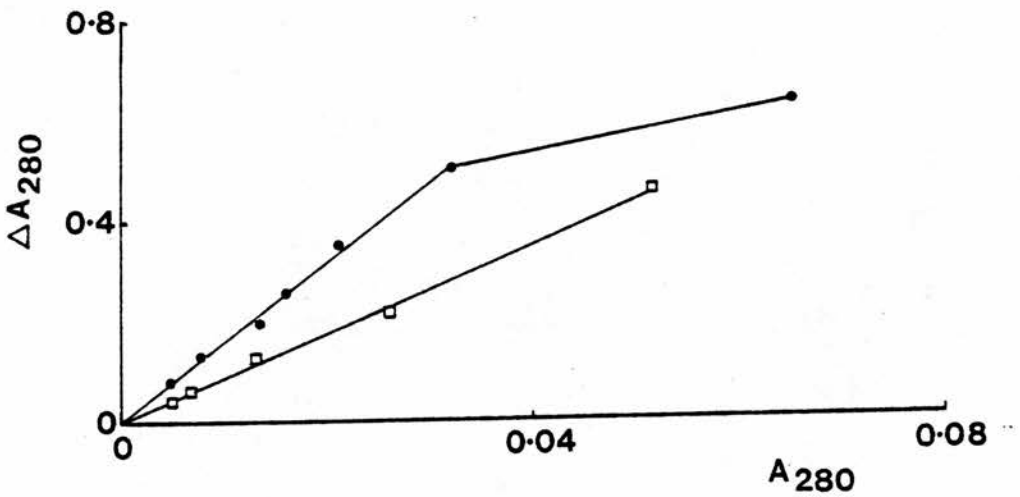


Fig. 24 Linearity of the haemoglobin assay. Samples (0·25 ml) containing various concentrations of the relevant enzyme were assayed by the routine assay method (Sect. 2.2). Enzyme concentration expressed as A_{280}.
●, peak I; □, peak III.

Chapter 4 ACTIVITY OF PEAK I AND III WITH HAEMOGLOBIN, A.P.D. AND Ac.Y.L.V.H.NH_2 AS SUBSTRATES

4.1 Linearity of the Assays

For the routine assay of chromatographic fractions it was necessary to determine the range of enzyme concentration, expressed as A_{280} of the sample solution, over which a linear response is found, in order that samples might be diluted to within the appropriate linear range.

4.1.1 Linearity of the Assay using Haemoglobin

The curves were prepared in a manner based upon the haemoglobin assay described in Sect. 2.2. Samples of peaks I and III, prepared by double chromatography on DEAE-Sephadex A50 and gel filtration on Sephadex G75 and freeze-dried as detailed in Sect. 3 were used. Stock solutions of lyophilizate of 1 mg/ml in water were prepared and centrifuged in an MSE bench centrifuge to remove any insoluble material. These solutions were diluted with water, the A_{280} of the diluted samples was determined in a Unicam SP 500 spectrophotometer and they were assayed in the usual way. The results are shown in Figure 24. For peak I the assay is linear for enzyme samples up to an A_{280} of 0·035, while for peak III it is linear up to an A_{280} 0·05.

Having established the range of linearity for peaks I and III, dilutions were generally made, where necessary, for the routine assay of all chromatographic peaks such that the A_{280} of fractions was around 0·01, well within the linear range.

4.1.2 Linearity of the Assay using A.P.D.

A fresh stock solution of 0·2 mg/ml in water was prepared using freeze-dried peak III material obtained by ion-exchange chromatography and/...

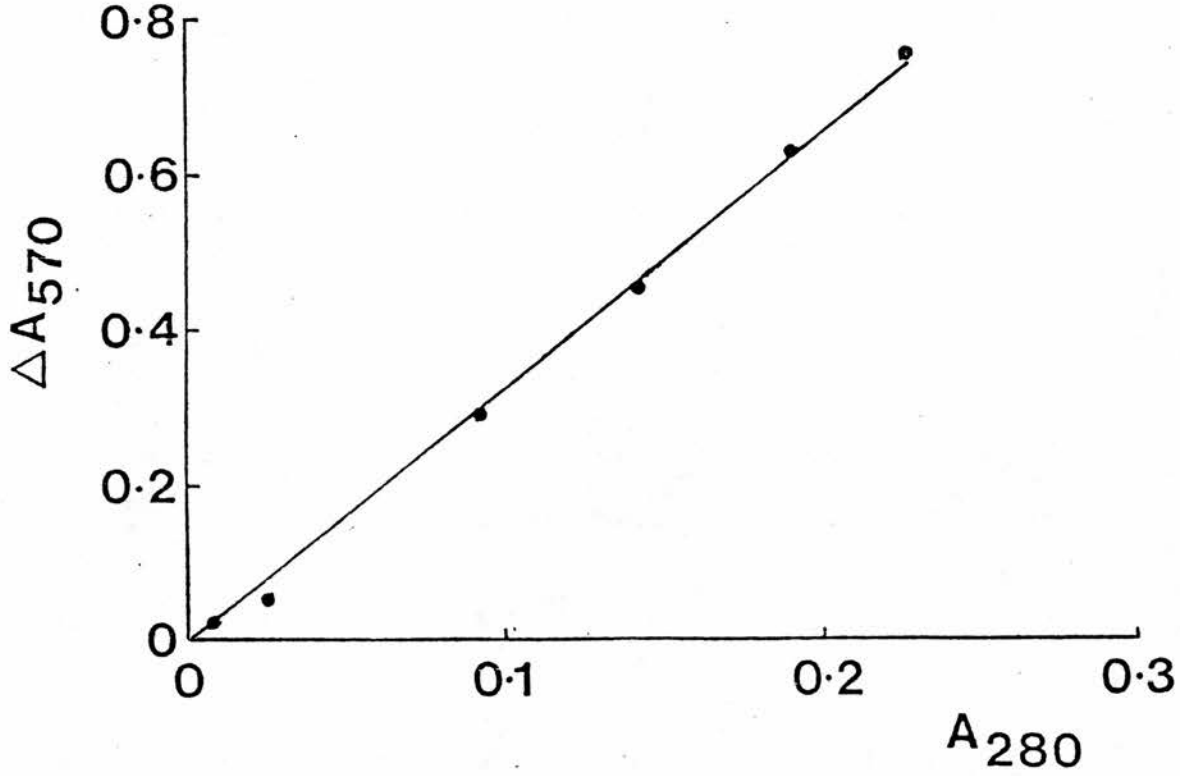


Fig 25 Linearity of the hydrolysis of A.P.D. by peak III. Samples (0·5 ml) containing various concentrations of peak III were assayed by the routine assay method (Sect. 2.3). Enzyme concentration is expressed as A_{280}.

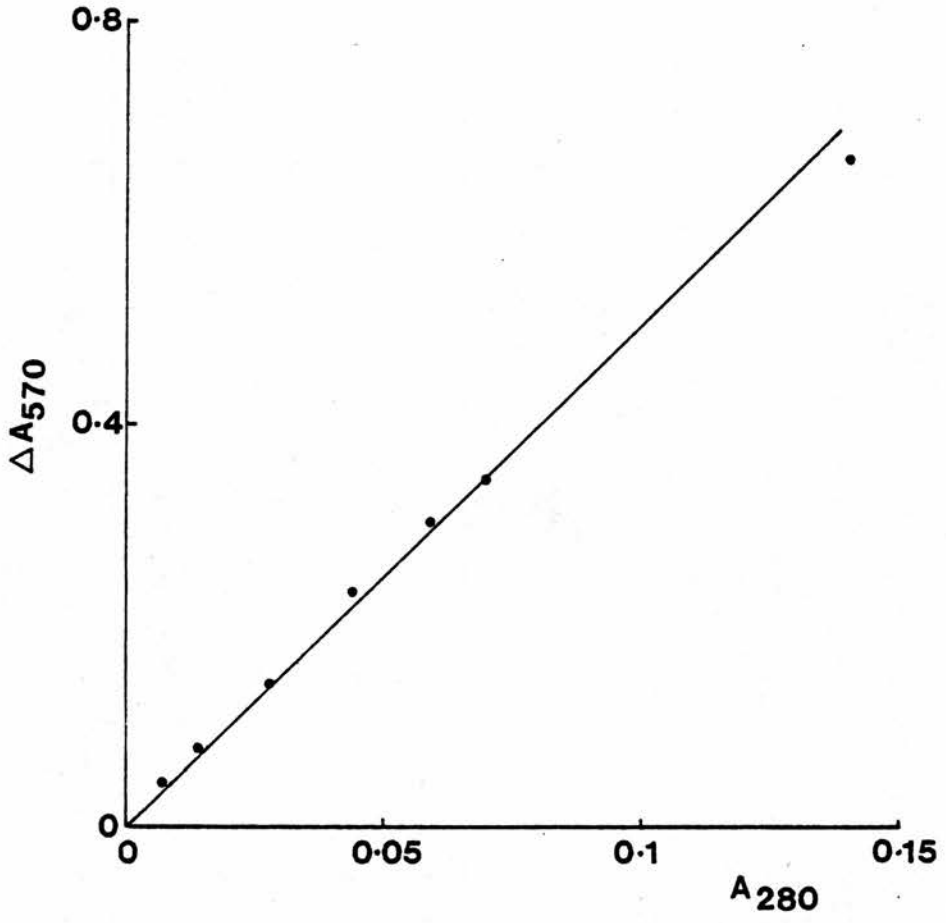


Fig. 26 Linearity of hydrolysis of Ac.Y.L.V.H.NH_2 by peak I. Samples (0·5 ml) containing various concentrations of peak I were assayed by the routine method (Sect. 2.4). Enzyme concentration is expressed as A_{280}.

and gel filtration as described in Sect. 3. Any precipitate present was removed by centrifugation in the MSE bench centrifuge. The A_{280} of this solution was measured and suitable dilutions were made with water. These samples were assayed by the method given in Sect. 2.3, the final pH being pH 2.3.

As may be seen from Figure 25, there is a linear response for enzyme solutions whose A_{280} is up to and including 0·23. Thus in work reported throughout this thesis, chromatographic fractions of most peaks were diluted such that the A_{280} fell within this range. Because of its lower activity against A.P.D., fractions of peak I were frequently assayed undiluted. In such cases, the concentration of HCl used in the assay had to be modified to produce the correct final pH, there being no buffer included in the assay.

4.1.3 Linearity of the Assay using Ac.Y.L.V.H.NH_2

A fresh solution of 0·2 mg/ml in water of peak I material, obtained by ion-exchange chromatography and gel filtration as described in Sect. 3, was prepared and centrifuged in an MSE bench centrifuge. The A_{280} of the solution was measured and a series of dilutions was made with water. The samples were assayed according to the routine method given in Sect. 2.4 , the final pH being pH 3·80.

The plot of ΔA_{570} as a function of enzyme concentration shown in Figure 26 indicates that there is a linear response for enzyme solutions in the range of A_{280} up to 0·15. Thus, in all work reported here, chromatographic fractions of peak I were diluted where necessary so their A_{280} fell within this range. As a rule, fractions of other pepsins were assayed undiluted because of their relatively low activity.

4.2 Effect of pH on hydrolysis of A.P.D. and Ac.Y.L.V.H.NH_2

The aim of this work was to investigate the feasibility of using these synthetic substrates in a differential assay in order to distinguish different pepsins in human gastric juice. Ryle and Auffret (1979) examined the activity of human pepsin and gastricsin (using the nomenclature of Tang, 1970), against the new substrate Ac.Y.L.V.H.NH_2 at 37° C, pH 2·07, and at an enzyme concentration in the incubation mixture of 2 - 3 μg/ml (i.e. a concentration of enzyme solution added of 4-6 μg/ml, equivalent to an A_{280} of about 0·006). These authors found that this concentration of pig pepsin C gave a rapid rate of hydrolysis of Ac.-Y.L.V.H.NH_2, and the studies using human pepsin and gastricsin were therefore performed under these conditions. Ryle and Auffret found that AcY.L.V.H.NH_2 was not hydrolysed by human pepsin or pig pepsin A when tested with 0·5 mM substrate, 2 μg of enzyme per ml, and at pH 2·0. However, these authors found that very slow hydrolysis of Ac.Y.L.V.H.NH_2 by human pepsin was detected when a ten-fold greater enzyme concentration was used. Therefore, in the work reported here, higher concentrations of enzyme were chosen in an attempt to determine whether or not the substrate is hydrolysed by both peaks I and III.

Furthermore, only one pH value was used by Ryle and Auffret for assay of human pepsin against Ac.Y.L.V.H.NH_2. In order to establish a differential assay it is clear that pH values should be chosen for each assay at which the different pepsins can be best distinguished. Thus, for the assay with Ac.Y.L.V.H.NH_2 conditions should be chosen in which the ratio of activities of peak I to peak III is highest, and for the assay with A.P.D., conditions should be chosen in which the ratio of the activity of peak III to that of peak I is highest. Therefore, for/...

for each synthetic substrate, the effect of pH on hydrolysis was determined using peaks I and III representing the main classes 'gastricsin' and 'pepsin'.

Auffret and Ryle (1979) studied the kinetics of these reactions using a Technicon autoanalyser. As a rule, estimation of initial velocities of reactions is best performed in this way rather than manually because of difficulties in obtaining results in a short time and because of manipulative errors in manual work. However, in this work, a detailed study of kinetics was not required. It was therefore felt that manual estimations would be satisfactory.

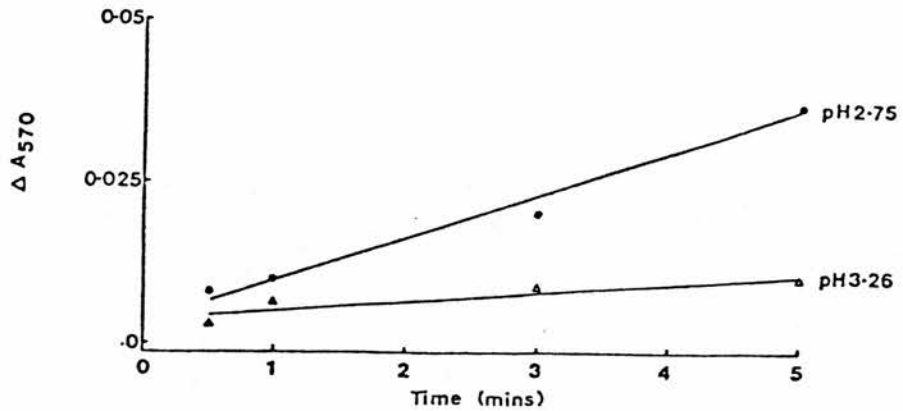
4.2.1 Effect of pH on hydrolysis of A.P.D.

The method used for this study was that of the routine assay (Sect. 2.3) except that the concentration of HCl was varied in order to vary pH. Also, the reactions were stopped at relatively short time intervals.

Samples of pepsins used were those of peaks III and I which had been prepared from human gastric juice by ion-exchange chromatography and gel filtration, as detailed in Sect. 3, and freeze-dried.

For the assays of peak III, a fresh stock solution of 0·1 mg/ml in water was used. Such a solution would have an A_{280} of about 0·13 , and would therefore fall within the linear range, as shown in Figure 25 . The enzyme solution was centrifuged in an MSE bench centrifuge before use.

The pH of the reaction mixtures was first determined using appropriate pepsin solutions which had been denatured by heating in a boiling water bath for 15 minutes. A sample of this solution was mixed/...



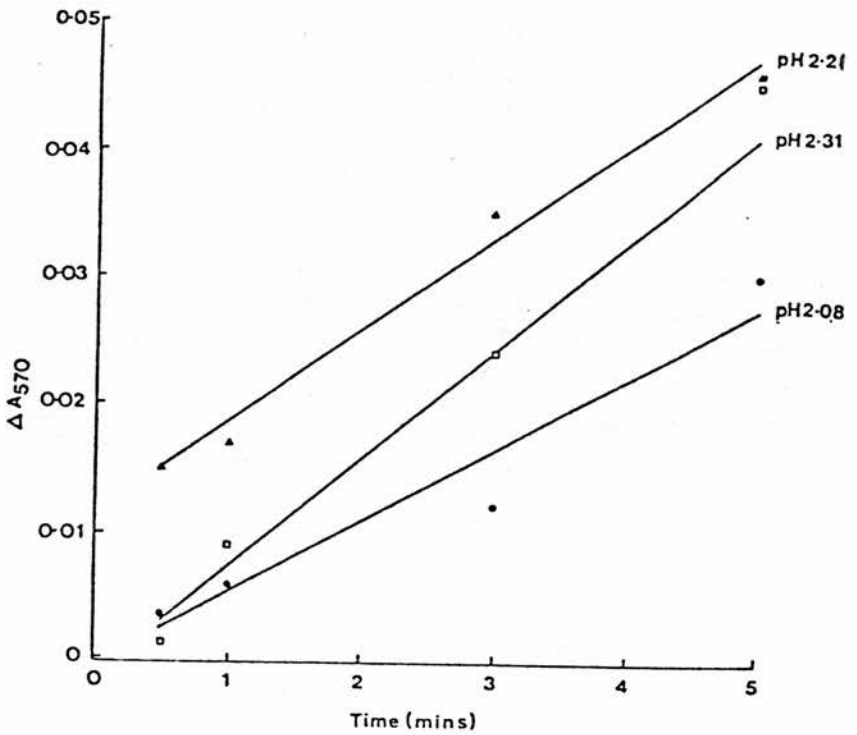


Fig. 27 The course of hydrolysis of A.P.D. by peak III at different pH values. Enzyme concentration in the assay mixtures was 0·05 mg/ml. Samples were assayed by the routine method (Sect.2.3).

mixed with half its volume of the required HCl solution, and half its volume of the stock A.P.D. solution. The resulting pH was measured in a Corning-Eel pH meter. On one occasion it was noted that at pH 1·6 a precipitate formed which re-dissolved when NaOH was added; this was probably A.P.D. This effect was not observed during any of the experiments reported here.

Assays were performed in the routine manner. For peak III, reactions were allowed to proceed for 30 secs, 1 min, 3 mins and 5 mins., after which ninhydrin was added and colour was developed as described in Sect. 2.3. Duplicates of active samples and of blanks were performed for each pH value.

Figure 27 shows a plot of ΔA_{570}, obtained by subtracting blank values from those of active samples, as a function of time. A range of pH 2·08-3·26 was used. The best fit for these lines was determined by linear regression analysis; the lines were not constrained to go through the point (0,0), nor was this point included in the analyses. The data shown in Figure 27 represent the mean value for ΔA_{570} for duplicates at each time point. For any given duplicate, the ΔA_{570} for each tube was never more than 2% away from the mean. Thus the data show very good agreement between duplicates. This being so, there were probably no manipulative errors involved in obtaining the data for pH 2·21, whose line clearly does not pass through the origin; it looks as though there is a "burst" in the 0-30 sec. region. Although there are sometimes good explanations for such a burst (e.g. the presence of two substrate components such that one component is hydrolysed more rapidly than the other) this cannot explain the situation here/...

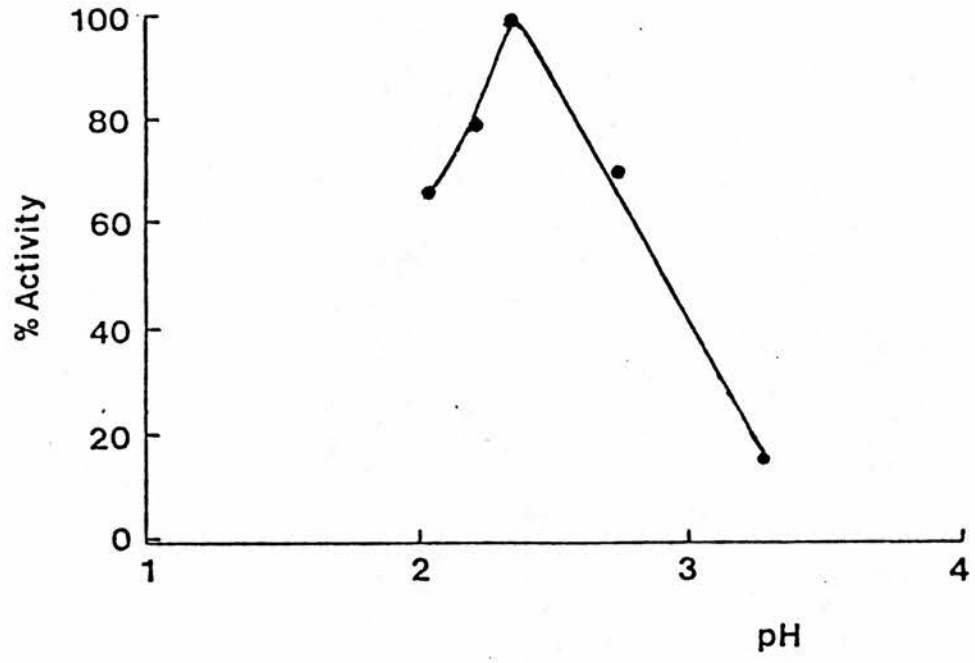


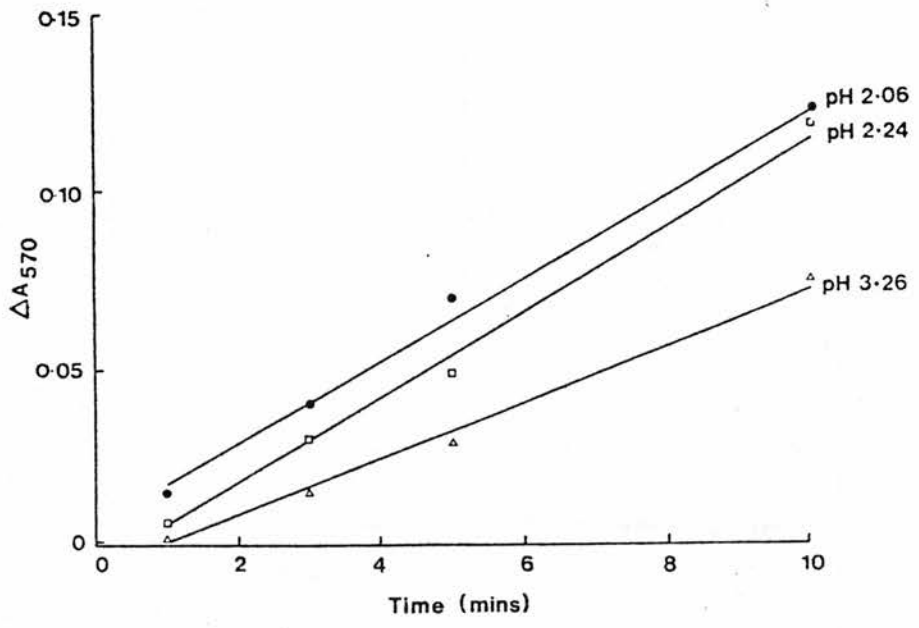
Fig. 28 The pH optimum for hydrolysis of A.P.D. by peak III.

here, because one would need to postulate such an effect only at pH 2·21, not at pH 2·08, 2·31 etc. It may be that the failure to pass through the origin is due to some constant error in the data for pH 2·21, such as a low absorbance in the reference cell, or high absorbance in the sample cell. Most of the data for this experiment were obtained on the same day, so it is not clear why such an effect should be confined to the data for pH 2·21. However, because the point of this series of experiments was to determine the approximate pH optimum for the routine assays it is the slope of the lines which is important, and therfore the data were considered adequate. Obviously, for more rigorous kinetic work, many more data would be required.

The specific activity of peak III, calculated as $\frac{\Delta A_{570}/ml/hr}{A_{280}}$, in this assay may be calculated from Figure 27. Based on the observation that the A_{280} of a 1 mg/ml solution of pepsin or gastricsin is 1·3 cm^{-1}, the specific activity at pH 2·21 is 8·7, which compares quite well with that of 10·7 shown in Table 7, Sect. 3, for a sample of peak III prepared from human gastric juice.

The line of greatest slope in Figure 27 was taken to represent 100% activity, and the slopes of all other lines were related to this. This was rather more convenient than plotting the actual slopes as a function of pH. A curve was obtained, as shown in Figure 28. From this, it appears that the optimum pH for the hydrolysis of A.P.D. by peak III is approximately pH 2·3.

The method used for investigation of the effect of pH on the hydrolysis of A.P.D. by peak I was similar. A solution of 0·4 mg/ml was prepared and centrifuged. Because of the relatively low activity of peak I/...



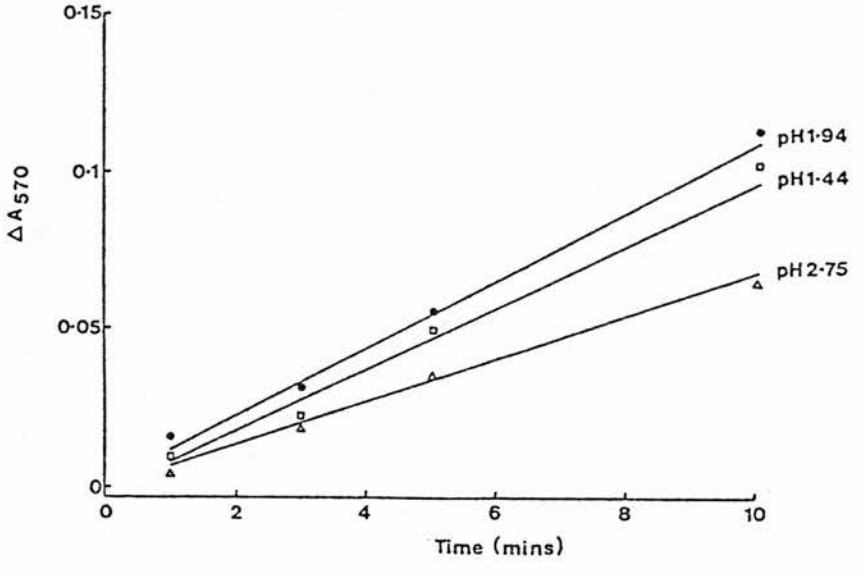


Fig. 29 The course of hydrolysis of A.P.D. by peak I at different pH values. Enzyme concentration in the assay mixtures was 0·2 mg/ml. Samples were assayed by the routine method (Sect. 2.3).

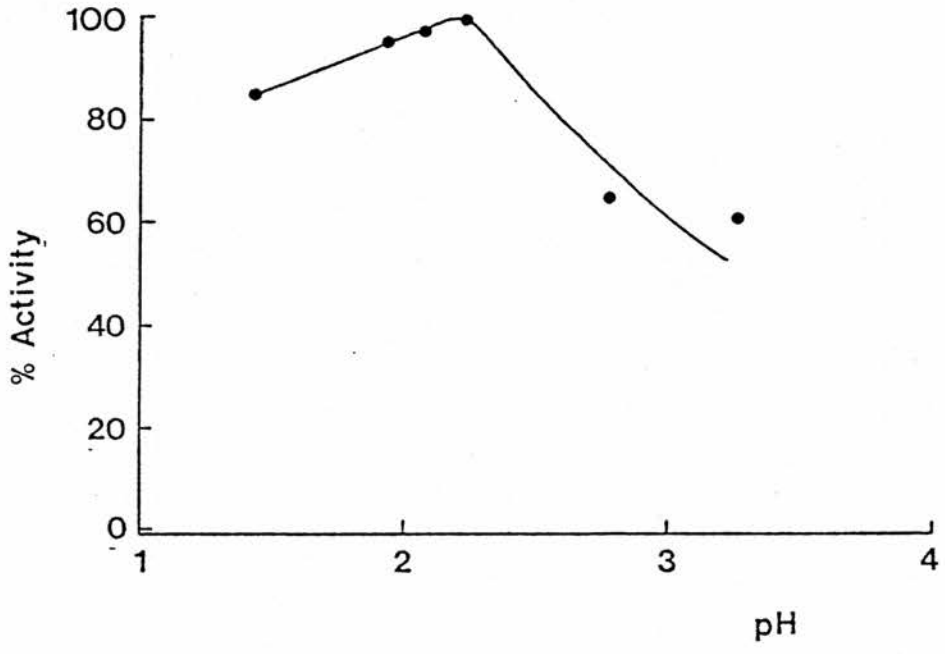


Fig. 30 The optimum pH for hydrolysis of A.P.D. by peak I.

peak I in this assay and the relatively short incubation times used, a higher concentration was necessary. Incubation times of 1,3,5 and 10 minutes were used. Figure 29 shows the course of hydrolysis at six different pH values. The data in Figure 29 were not all obtained at the same time. On some occasions data for particular pH values were rejected because of known errors in timing, pipetting etc. Such experiments were repeated at a later date along with experiments using a pH value at which good data had previously been obtained. By comparison of the slopes of lines for the pH value common to both sets of experiments, the new data could be fitted in. Taking the slope at pH 2·24 as representing 100% activity, Figure 30, showing % activity as a function of pH, was constructed. In this case the optimum pH for hydrolysis appears to be around pH 2·25.

Comparison of Figures 28 and 30 shows that the fall-off in activity for peak III against A.P.D. is rather more marked than that of peak I. Whether or not any significance can be attached to this on the basis of the data presented here is uncertain. It is known that different pepsins have different stabilities at alkaline pH values (Walker & Taylor 1979; Becker & Rapp 1979), although they would not be expected to suffer inactivation at pH values as low as pH 3·2-3·5.

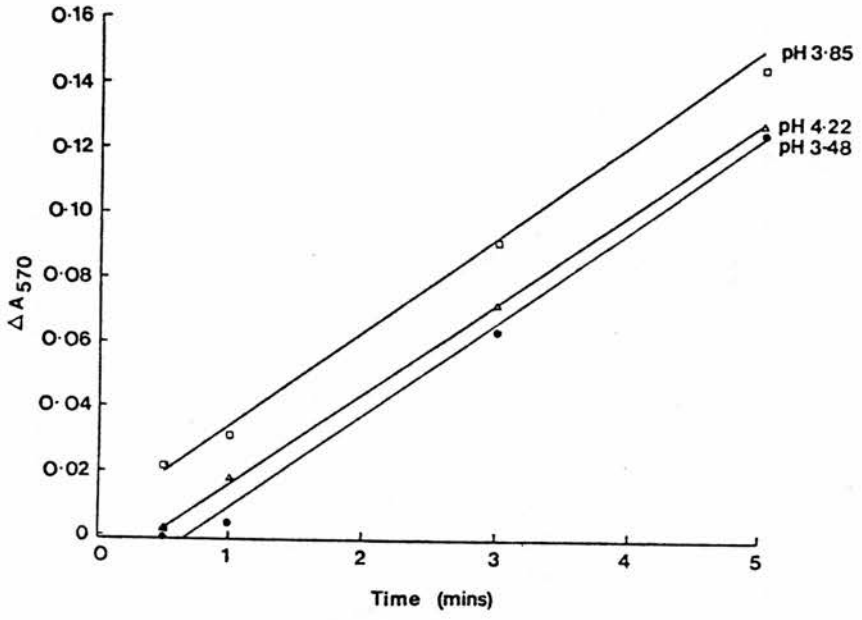
The specific activity of peak I can be calculated from the data shown in Figure 29 for pH 2·24. This value is 3·0, which is considerably higher than that of 0·74, at pH 2·3, shown in Table 7. It may be that in this assay linearity is not maintained, so assay over one hour gives a misleadingly low specific activity. If this is so, it may be that the specific activities for the action of peak I on A.P.D. quoted throughout this thesis are rather low.

Given that the activity of peak III against A.P.D. appears to decrease more rapidly than that of peak I as pH increases, it may be that at slightly higher pH values (pH 3·5-4) A.P.D. could be used to distinguish the peaks by virtue of activity of peak I and lack of activity of peak III in this assay. However, the measured A_{570} values in such an assay scheme may be too low for reliability.

4.2.2 Effect of pH on hydrolysis of Ac.Y.L.V.H.NH_2

The method of assay used was that given in Sect. 2.4, except that the substrate was dissolved in buffers of varying pH values. These buffers were prepared from citric acid and NaOH, and all were 0·05 M in citrate ions. In their work, Auffret and Ryle (1979) maintained constant ionic strength by the addition of NaCl. However, it was felt that in the differential assay of gastric juice ionic strength could not be standardised in this way. If gastric juice samples needed to be considerably diluted before assay, then the fact that they initially had different ionic strengths would be unimportant. However, it may be that for the assay using Ac.Y.L.V.H.NH_2, dilution of gastric juice would be undesirable because of the low concentration of peak I. This point is explained in greater detail in Sect. 5. Furthermore, work by Hollands and Fruton (1968) indicated that changing ionic strength did not affect kinetic parameters for the hydrolysis of the synthetic substrate Z-His-Phe-Phe-OEt. Therefore, it was felt that, in the experiments described here, adjustment of ionic strength was unnecessary.

In these assays using peak I, the stock solution used was 60 μg/ml in water/...



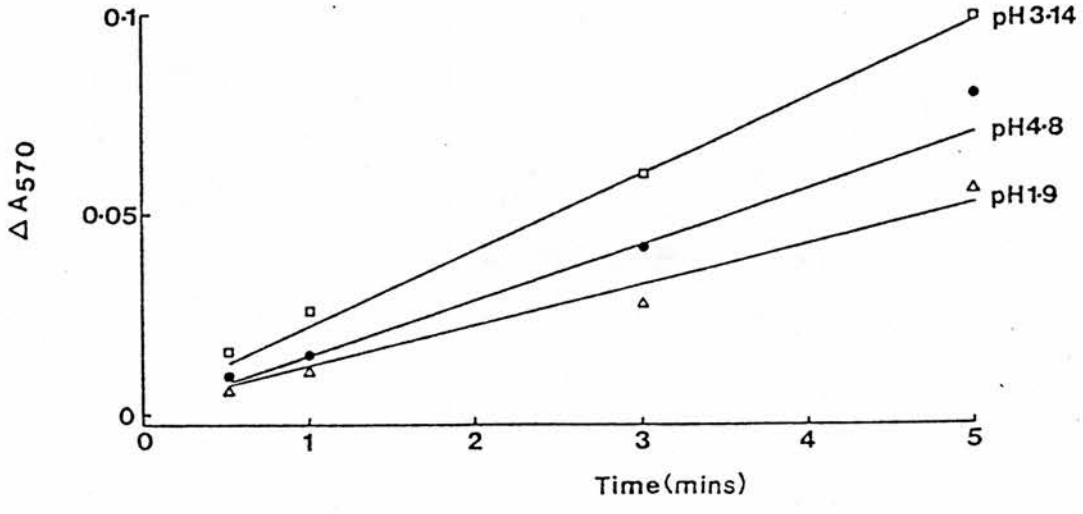


Fig. 31 The course of hydrolysis of Ac.Y.L.V.H.NH_2 by peak I at different pH values. Enzyme concentration in the assay mixtures was 30 μg/ml. Samples were assayed by the routine method (Sect. 2.4).

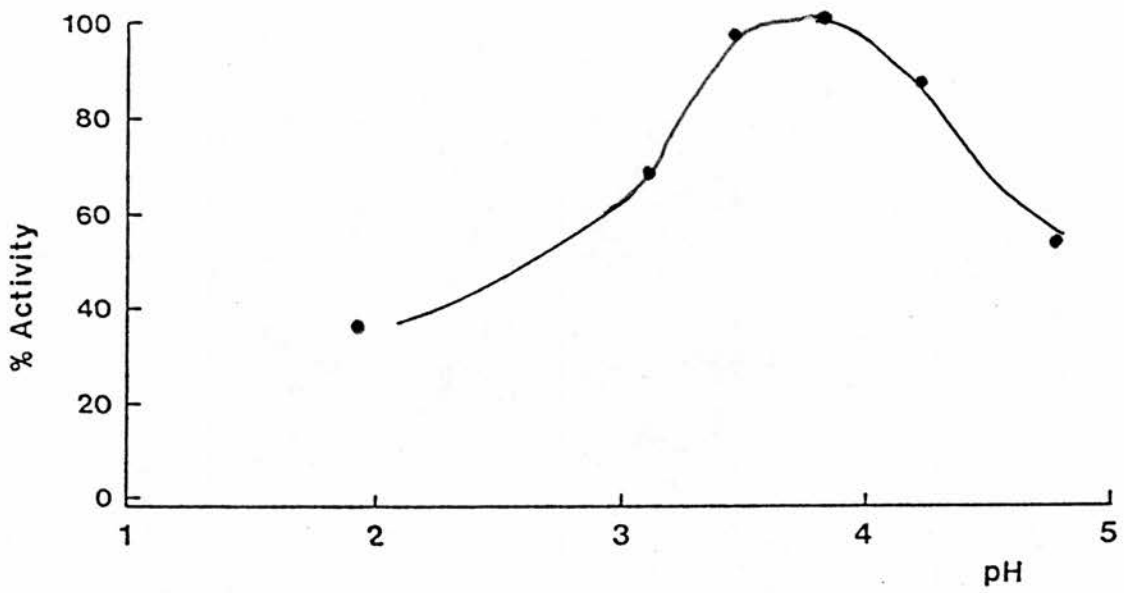


Fig. 32 The optimum pH for hydrolysis of Ac.Y.L.V.H.NH_2 by peak I.

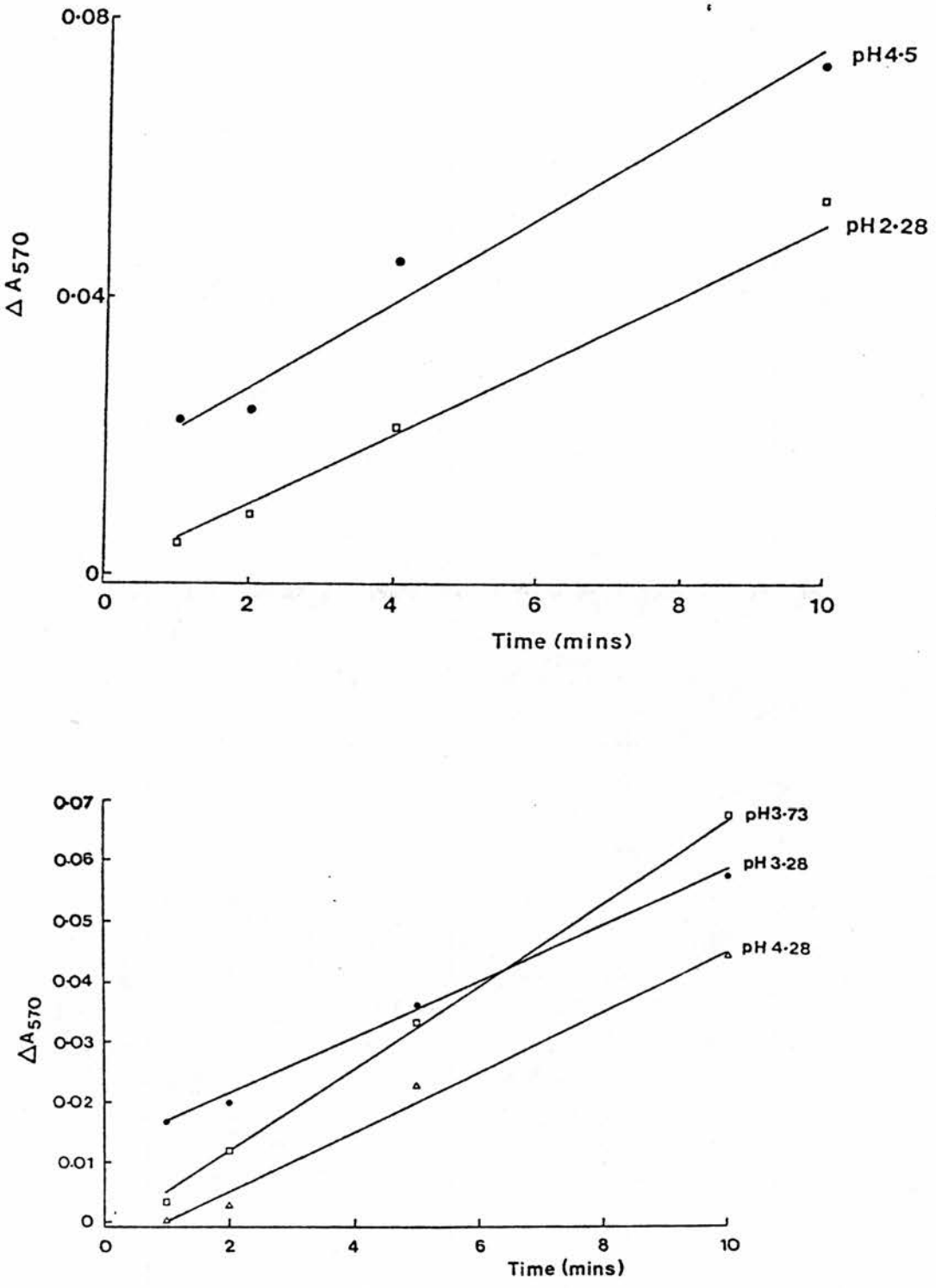


Fig. 33 The course of hydrolysis of Ac.Y.L.V.H.NH_2 by peak III at different pH values. Enzyme concentrations in the assay mixtures was 0·2 mg/ml. Samples were assayed by the routine method (Sect 2.4).

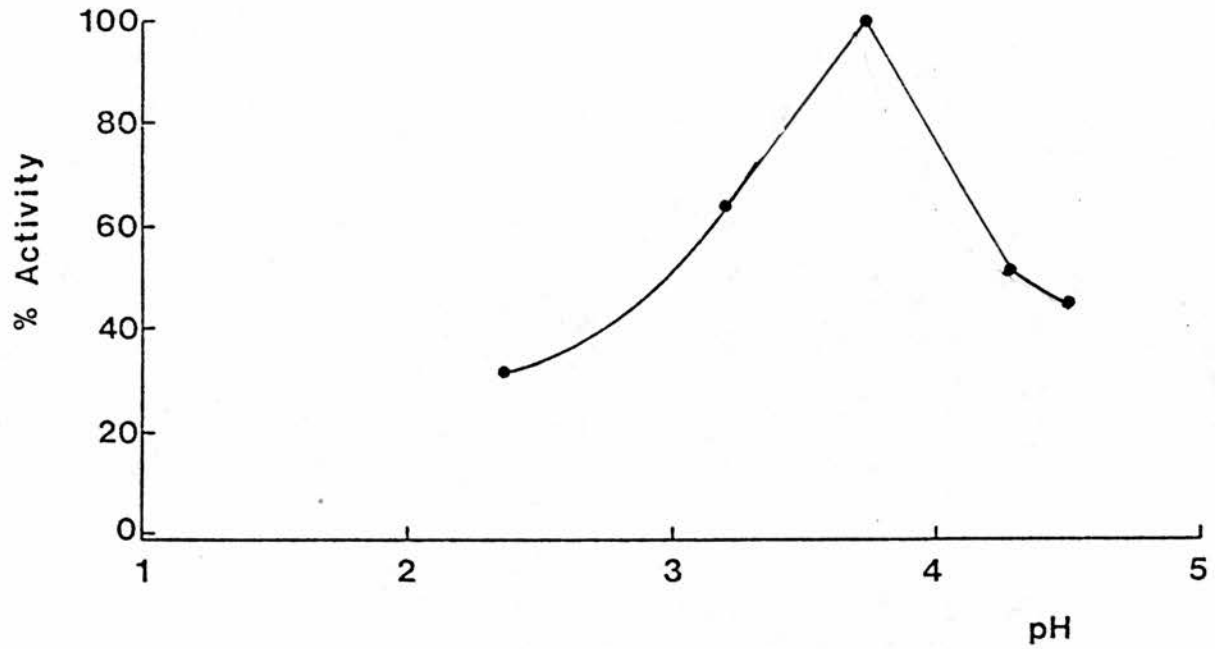


Fig. 34 The optimum pH for hydrolysis of Ac.Y.L.V.H.NH_2 by peak III.

in water. Reactions at the different pH values were stopped after 30 secs., 1 min., 3 and 5 mins. Figure 31 shows plots of A_{570} as a function of time for six pH values. The data shown in Figure 31 were not all obtained on the same occasion. Allowance for this fact was made in the manner described in Sect. 4.2.1. From the slopes of the lines in Figure 31 activities are expressed as a percentage of that at pH 3·85, as explained in Sect. 4.2.1. The plot of % activity as a function of pH is shown in Figure 32, from which it can be seen that the pH optimum is about pH 3·8. This was the pH at which all routine assays using Ac.Y.L.V.H.NH_2 described in this work were performed since this is the pH of maximum sensitivity of the assay.

From Figure 31 the specific activity at pH 3·85 can be calculated as 15.5, which compares well with that of 14·3, obtained at pH 3·80, as shown in Table 7.

For the experiments to determine the pH optimum for hydrolysis of Ac.Y.L.V.H.NH_2 by peak III, the stock enzyme solution used was 0·4 mg/ml. Figure 33 shows the time course of hydrolysis at five different pH values, the reactions being stopped after 1,3,5 and 10 minutes. The optimum pH was calculated from the slopes of these lines, as described in Sect. 4.2.1. The pH-activity curve thus obtained is shown in Figure 34, and the optimum appears to be around pH 3·7.

As may be seen from Figure 33, the lines for pH 3·28 and pH 4·5 do not pass near or through the origin. This effect has been discussed for the assay with A.P.D. (Sect. 4.2.1), and similar considerations may apply here.

Comparison/...

Comparison of Figure 32 and 34 indicates that the ratio of activity of peak I to that of peak III is highest at about pH 4·0 which would therefore be the pH of choice in any differential assay. As mentioned above, routine assays were performed at pH 3·8, this being the pH of maximum hydrolysis, and thus the best pH for detection of enzymes in chromatographic fractions etc.

The specific activity for hydrolysis of Ac.Y.L.V.H.NH_2 by peak III may be calculated from Figure 33; at pH 3·73, this appears to be 0·56, which compares well with that of 0·6, obtained at pH 3·8, shown in Table 7.

Chapter 5 DISCUSSION

Most of the higher animals secrete more than one proteinase of the pepsin type. The existence of more than one pepsin in the gastric juice of humans was clearly demonstrated when pepsin and gastricsin were first isolated and shown to have different activities against haemoglobin and A.P.D. Elucidation of the amino acid compositions of human pepsin and gastricsin (Mills and Tang, 1967) and of partial amino acid sequences (Sepulveda et al 1975) confirmed that they are distinct proteins. The possible role of pepsins in the aetiology of gastric and duodenal ulceration has attracted considerable interest for some years.

Of those studies concerned with the analysis of gastric pepsin secretion and disease, the majority have involved the assay of total peptic activity, e.g. by the assay of proteolytic activity of unfractionated gastric juice against various substrates, haemoglobin being the most popular. Estimation of the levels of individual pepsins would be much more informative, and would be greatly improved by the use of substrates specifically designed for the purpose. If any differential assay is to be applicable to unfractionated gastric juice, thorough characterisation of gastric juice components is essential.

The fractionation of human gastric juice on DEAE-Sephadex A50 reported here represents the best separation of pepsins so far published. Roberts and Taylor (1978a) have published details of the preparation and purification of four human pepsins using DEAE-cellulose with preliminary evidence of the existence of two other pepsins. The/...

The method used a number of chromatographic stages, using different conditions of pH and gradients of NaCl concentration. Overall, this method appears rather inconvenient and laborious. Particular difficulty was encountered in preparing pepsin 5 free from pepsin 3. By using DEAE-Sephadex A50 under the conditions described here, relatively good separation of seven clearly-defined fractions has been obtained.

The status of peak Ia is very interesting. This fraction appears to be the largest of the fractions studied here, having a molecular weight of about 45,000, as determined on SDS-polyacrylamide gel electrophoresis; by this criterion peak Ia was probably homogeneous. Obviously homogeneity could not be checked by staining for activity. Gel filtration indicated a much lower molecular weight. Under the assay conditions used, this peak showed no activity against either of the synthetic peptides, A.P.D. or Ac.Y.L.V.H. NH_2, and on most occasions showed no activity against haemoglobin. It should be noted that failure to show activity against haemoglobin may reflect the assay technique. Thus, it is possible that hydrolysis occurs but that the fragments are precipitated by trichloroacetic acid (T.C.A.), or perhaps the T.C.A. - soluble fragments do not contain tyrosine or tryptophan and are therefore not detected. The peak Ia was detected in all gastric juices examined. In the pig, pepsin B has been well-characterised (Ryle and Porter, 1959; Ryle 1965). This enzyme shows little or no activity against haemoglobin, but is twice as active against A.P.D. as is porcine pepsin A. Furthermore, pepsin B is able to clot milk at pH 5·6. It is interesting to note that during chromatography on DEAE-cellulose at pH 4·0 pepsin B is the first pepsin to emerge, while peak Ia is the first peak to emerge on DEAE-Sephadex A50 at pH/...

pH 4·0. Thus, there are some similarities between peak Ia and porcine pepsin B, but the failure of peak I a to show appreciable activities in any of the assays, including the clotting of milk, would suggest that this material is not an equivalent of porcine pepsin B. No such equivalent has yet been convincingly demonstrated in human gastric juice. Overall there is little evidence that peak Ia is pepsin-like. As has already been mentioned, the retardation of this material on Sephadex G75 may indicate that it is a proteoglycan (Sect. 3.7.1.). Although there are some similarities in amino acid composition shown for peak Ia and the pepsins, there are also several differences; it should be emphasised that these analyses represent single determinations and may be somewhat unreliable.

It appears that peak Ia has not been located and studied by any previous authors, although it is noticeable that a small peak was eluted at low NaCl concentration in the chromatogram of human gastric juice on DEAE-cellulose at pH 5·3 shown by Roberts and Taylor (1978 a). These authors did not investigate this material. The question of whether or not peak I a is a proteinase remains open. It is possible that it has a different range of specificity from any pepsin so far examined; investigation of the action of peak Ia on protein substrates such as ovalbumin and the B-chain of oxidised insulin might reveal such specificity.

Peak I appeared in all gastric juices studied. Virtually all the evidence presented here regarding order of elution, electrophoretic mobility, proteolytic activity, amino acid analysis and abundance in gastric juice indicates that this enzyme is equivalent to gastricsin (Richmond et al 1958) and pepsin 5 (Etherington and Taylor 1969). However, Chiang et al (1966) found that gastricsin had/...

had no activity against A.P.D. The data presented here are in direct contradiction to this. Peak I, which was shown to be electrophoretically homogeneous, retained some measurable activity against A.P.D. However, comparison of Tables 2 and 5 shows a decrease in specific activity of peak I against this substrate as purification proceeded. This may indicate increasing separation of peak I from a contaminant which was actually the cause of the activity. Given the clear separation of peak I from the other pepsins during electrophoresis, it is difficult to envisage the possible nature of any such contaminant. On this basis it appears likely that this activity is a genuine property of peak I. It is interesting to note that Samloff (1969) has described two zymogens whose enzymes have gastricsin-like properties; there was no evidence from any of the work reported here for the existence of more than one enzyme of this type.

The isolation of peak II is also very interesting. This material is intermediate in electronegativity between peaks I and III, as shown by ion-exchange chromatography and electrophoresis. In their experiments, Etherington and Taylor (1969) found an electrophoretic zone (zone 4) between zones 3 and 5, but this zone was actually due to a complex between pepsin and the pepsin inhibitor released during pepsinogen activation. It is highly unlikely that peak II would represent any such complex because during the course of chromatography samples were at pH 4 for 2 -3 days, under which conditions one would expect pepsin inhibitor to be digested. It should be noted that, on agar gel electrophoresis, the mobilities of peaks II and III are very similar, so it may be that the presence of peak II is masked in the gels published by Taylor and his co-workers; the poor separation of peaks under the conditions/...

conditions used by these authors may produce the same effect.

Thus it appears that the existence and characterisation of peak II has not previously been reported. Peak II appears to be inactive against Ac.Y.L.V.H.NH_2 and active against A.P.D. and haemoglobin. These facts, together with the amino acid analyses, indicate that peak II is a "pepsin-like", rather than "gastricsin-like", enzyme.

It is clear from the data given here that peak III is analagous to pepsin 5 described by Taylor and co-workers. It is the most abundant pepsin in gastric juice and was present in all gastric juices examined.

Peak IV occurred in all gastric juices examined except for that of one 51 year-old female with duodenal ulcer. In all cases where peak IV was located it occurred as a shoulder on the main peak, peak III. The data for electrophoretic mobility on agar gel (Table 6) indicate that peak IV has a somewhat similar mobility to pepsin 3a. Etherington and Taylor (1969) in a study of 50 gastric juices reported that the frequency of occurrence of pepsin 3 a is unknown because it is easily masked by pepsin 3. The improved technique detailed here reveals that this material, assuming it is analagous to peak IV, occurs in virtually all gastric juices. The chromatograms of Roberts and Taylor (1978 a) clearly show that there seems to be very low levels of pepsins 3a in gastric juice, and these authors made no attempt to isolate it. However, in this thesis it can be seen that appreciable levels of peak IV were routinely detected - this work thus represents the first successful attempt to purify milligram quantities of this protein. It has been obtained in a homogeneous form, as shown by polyacrylamide gel electrophoresis and SDS-polyacrylamide gel electrophoresis. The amino acid composition of peak IV is broadly similar to that of peak III although their specific activities help to confirm that they are distinct/....

distinct proteins. The ratio of glx:asx and the specific activities against the three substrates indicate that peak IV belongs in the "pepsin" group as opposed to the "gastricsin" group.

The separation and isolation of peaks V and VI proved very difficult. Extremely small quantities were isolated. Electrophoresis in agar gels indicated that the mobility of peak V is very similar to that of pepsin 2 (Taylor's nomenclature). On the basis of their order of elution from ion- exchangers it appears that peak VI may be analagous to pepsin 1, although Roberts and Taylor (1978a) have reported the detection of a new enzyme, named "slow pepsin 1" which has an electrophoretic mobility intermediate between pepsins 1 and 2. In the polyacrylamide gels reported here there is no evidence of three zones in this region. A typical chromatogram is shown in Figure 4. The positions of elution of peaks V and VI were somewhat more variable than those of the other enzymes. Although Figure 4 shows 750 ml. of eluting buffers being used, on several occasions the chromatogram was extended beyond this, yet there was no evidence of any other peaks. Thus, peak VI could be equivalent to either pepsin 1 or "slow pepsin 1". As discussed in the Introduction, many authors have failed to locate these minor enzymes. Thus, the chromatographies presented here show the best separation so far published of these enzymes from the major pepsins. It is particularly interesting to note that Roberts and Taylor were able to isolate milligram quantities of pepsin 1 and up to 200 μg of pepsin 2 from a single gastric juice but they could not obtain such quantities of pepsin 3a, whereas the situation is reversed in the work reported here. The reasons for this anomaly are unclear.

As far as could be determined, both peaks V and VI showed activity against haemoglobin and A.P.D. Results of assays with Ac.Y.L.V.H.NH_2 are/...

are more equivocal, as has been discussed (Chapter 3), possibly because of very low protein concentrations. The amino acid compositions of peaks V and VI show some quite remarkable differences from those of the other enzymes, especially that peaks V and VI contain only two half-cystine residues per molecule. Possible explanations for the high content of leucine have already been discussed (Sect. 3.6). Both peaks V and VI are relatively high in the basic amino acid residues. These proteins are clearly more acidic than the others being considered here, so peaks V and VI must presumably have relatively high ratios of glutamate:glutamine and/or aspartate:asparagine, or possibly they contain other non-amino acid, acidic groups, e.g. phosphate groups. It is noticeable that while peaks V and VI have such differences from the other enzymes they are very similar to each other.

One potential difficulty in the study of proteolytic enzymes is that of autolytic degradation. Foltmann (1964) found that when chromatographically pure A-rennin was incubated at pH 3·5 for 6 hours, at 25° C, a new peak was detected on DEAE-cellulose chromatography, while the height of the original peak simultaneously decreased. Of particular importance is the fact that the new material, formed by autolysis of the original protein, retained proteolytic activity. Also, Kageyama and Takahashi (1980) found that acid treatment of human cathepsin D increased its anodal mobility. The new material retained activity and apparently had the same molecular weight as the original; it was suggested that the change in mobility was due to structural changes caused by autodigestion. It may be noted, of course, that it would be difficult to differentiate, in the experiments of Kageyama and Takahashi, between autolysis of fully-formed enzyme and the final stages/....

stages of activation of zymogen. Activation of pig pepsinogen occurs in more than one step involving limited proteolysis (Dykes and Kay 1976; Kay and Dykes 1976, 1977; Christensen et al 1977). Therefore it appears that there are cases in which proteolytically-active fragments can be formed after autolysis. Whitecross et al (1974) suggested that pepsin 1 is a product of autolysis; some criticisms of this work have already been discussed (p. 23). Also, Tang (1979) suggested that only "pepsin" and "gastricsin" have been structurally defined and that the remaining isozymes are likely to be degradative products.

Thus, it could be postulated that a limited degradation of, for example, peak III might occur which leaves the enzyme active but which changes its behaviour on ion-exchange resins and thus yields a new peak or peaks. However, the experiments reported here indicate that incubation of gastric juice at pH 4·0 for three days did not cause any changes in peaks heights. Therefore it would appear that autolysis during chromatography, which generally lasted three days, is not responsible for the existence of any of the peaks described here. Similarly, it was shown that incubation of peak III at pH 1·2, the pH of collection of many of the gastric juices, did not cause the appearance of other peaks. The inter-relationships of the other peaks is less clear. Peak I is clearly distinct from the other enzymes, by physical (alkali, heat, acid stability, amino acid composition) and biochemical (specific activities) criteria, and is unlikely to be a degradative product. Also, peak II seems to be distinct in its behaviour with Ac.Y.L.V.H.NH_2. Peak IV is quite similar to peak III in amino acid composition, molecular weight and acidic nature. As explained, it seems that autolysis of peak III does not yield other fractions. It is unlikely, given their relative/...

relative abundancies that autolysis of peak IV would be responsible for the existence of peak III. Peak V and VI are closely related but comparison of their amino acid compositions makes it unlikely that either is a degradative product of the other.

Thus it appears that the chromatographic fractions obtained here are distinct enzymes as opposed to autolytic products. The isolation and characterisation of their zymogens would be a most useful step in confirming this. If this conclusion is correct, it would cast doubt on a lot of older work in this field.

A total of five gastric juice samples were obtained from healthy volunteers and chromatographed separately, as were six samples from duodenal ulcer patients. In all cases, the elution profiles were qualitatively very similar. Peaks V and VI were not always obvious in the same gastric juice; with their very low concentration it was generally difficult to distinguish these peaks. These data are not sufficient to draw any firm conclusions about any relationship between the occurrence of individual pepsins and ulceration, but as far as can be seen there was no such relationship. It is clear that chromatography is not a very good technique for this type of study, being unreliable for quantitation, because of possible freeze-drying and other mechanical losses and because of uncertainty in estimating amounts of protein in the peaks. Also, chromatographic procedures are extremely slow if a large number of gastric juice samples are to be analysed. Enzymatic assay may offer the best method for assessment of individual pepsins.

The substrate Ac.Y.L.V.H.NH_2 was designed by Auffret & Ryle (1979) as a substrate for pig pepsin C, the equivalent of human gastricsin. It was suggested by Auffret (1977) that the use of Ac.Y.L.V.H.NH_2 would allow/...

allow assay of gastricsin, while A.P.D. would allow assay of pepsin in unfractionated gastric juice. Simultaneous equations were formulated to account for any reactivity of pepsin with Ac.Y.L.V.H.NH_2 and of gastricsin with A.P.D.

In the work described here, samples of peaks I and III were used to determine the influence of pH on the hydrolysis of the syntheticsubstrates. It was found that both enzymes were active against both substrates, albeit with different specific activities. Electrophoresis indicates that the enzyme samples were homogeneous. If the activity of peak I against A.P.D. could be explained by contamination, one would need to postulate that such a contaminant is sufficiently similar to peak I that it was not removed during two ion-exchange steps and one gel filtration step. Given the electrophoretic separations it seems unlikely that any of the other, "pepsin-like" enzymes reported here would be the cause of such contamination. It is probable that peak I has intrinsic activity against A.P.D., and similarly that peak III has intrinsic activity against Ac.Y.L.V.H.NH_2. For either substrate, the optimum pH values for the two enzymes were very similar, although it would be possible to select conditions under which the discrimination between enzymes could be maximised.

At the time of Auffret's work, most of the various electrophoretic bands identified in gastric juice had not been isolated and characterised. It was possible that they represented residual zymogen or enzyme-inhibitor complexes etc. However, the work presented here indicates that there are at least six active fractions which have been isolated and apparently represent discrete enzymes. The evidence indicates, furthermore, that peaks I and III are not the only fractions which hydrolyse/....

hydrolyse A.P.D. and Ac.Y.L.V.H.NH_2. Thus, peak IV appears to hydrolyse both substrates while peak II hydrolyses A.P.D.; it may be that peaks V and VI behave in a similar way.

In the assay of gastric juice using A.P.D., a dilution of the gastric juice of five-fold or so was generally necessary in order to ensure that the change in absorbance at 570 nm being measured fell within an acceptable range. In such a case, it can be shown that the peak I is so diluted that its effect in the assay would be negligible. However, for some individual gastric juices, if one estimates the total A_{280} under peak I, it can be shown that dilution would not be required for the Ac.Y.L.V.H.NH_2 assay. In that case, activity of the other peaks against Ac.Y.L.V.H.NH_2 would have a significant effect on the result, leading to a further complication.

Despite these problems, it might be feasible to devise a differential assay which would give the levels of "total pepsin-like enzymes" and "total gastricsin-like enzymes" in unfractionated gastric juice. However, this would be of limited value in any investigation of the role of individual pepsins in ulceration. The work of Taylor and his colleagues has indicated that pepsin 1 is the pepsin most frequently associated with peptic ulceration, (e.g. Taylor 1970). It appears that peak VI may be the equivalent of pepsin 1. Data in Table 2 indicate that peak VI may have activity against all three substrates used. It would be virtually impossible to detect changes in the concentration of this enzyme in gastric juice using the synthetic substrates, especially in the presence of large amounts of peaks I-IV.

It is clear that the assay of six separate proteinases with only two synthetic substrates is impossible. The most fruitful approach to this problem would be the design and synthesis of novel substrates specifically for each enzyme. This may be difficult, as preliminary results obtained by Roberts and Taylor (1979) indicate that pepsins

1,/...

1,2 and 3 all attack the B-chain of oxidised insulin at the same bonds.

It may be that immunological techniques will yield sensitive assays for the individual pepsins. Quantitation of immunoelectrophoretic techniques would probably be somewhat subjective, as are other electrophoretic and isoelectric focussing methods. Possibly, radioimmunoassay could be a useful technique. A competitive binding, double antibody radioimmunoassay has been developed for the separate and specific determination of the human pepsinogen group, PG I (Samloff & Liebman 1974). Subsequently, Samloff (1982) has developed a new radioimmunoassay for the PG II group, and an improved R.I.A. for PG I; pepsinogens were isolated from gastric mucosa rather than urine. PG I did not react in the assay for PG II and vice-versa. Samloff is currently studying possible clinical usefulness of the assay of serum PG I and PG II.

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